



CD4 T Cells Regulate Adenovirus Vector-Elicited Cellular and Humoral Immune Responses

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CD4 T Cells Regulate Adenovirus Vector-Elicited Cellular and Humoral Immune Responses

A dissertation presented

by

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to

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in partial fulfillment of the requirements

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Abstract

The processes that regulate viral vector vaccine-elicited cellular and humoral immune responses remain poorly defined. Thus, in this thesis, the role of CD4⁺ T cells – master regulators of adaptive immunity – in modulating adenovirus (Ad) vector-elicited cytotoxic CD8⁺ T cell responses and transgene-specific antibody responses was investigated. CD4⁺ T cell help is critical for the induction of CD8⁺ T cell and antibody responses, but the mechanisms and timing of help to each of these two arms of the immune system are distinct. CD4⁺ T cell help is required immediately and continuously for one week to drive functional CD8⁺ T cell effector differentiation and prevent dysfunction. Elevated signaling via PD-1, decreased IL-2 signaling, and increased non-canonical NFAT signaling all appear important for driving this CD8⁺ T cell dysfunction and impairing effector functionality. Absence of CD4⁺ T cells at the time of Ad vector immunization prevents the development of antigen-specific antibody responses. However, if the CD4⁺ T cell population is allowed to recover then fully functional antigen-specific antibody responses develop without the re-administration of antigen. Thus, CD4⁺ T cell help is absolutely required for the development of antibody responses following Ad vector immunization, but, intriguingly, help can be provided at a time separate from initial antigen exposure. Collectively, CD4⁺ T cell help and the appropriate timing of this help are critical for the generation of optimal CD8⁺ T cell and antibody responses following Ad vector immunization. These data advance our understanding of how CD4⁺ T cells regulate Ad vector-elicited cellular and humoral immune responses, and may improve rational vaccine design.

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Dedication: To my wife, Rebekkah, and my daughters, Zoe and Pippa, thank you for always keeping me grounded and focused. Thank you for your endless support and patience. To my parents, Ronald and Lois, and to my ever-growing family, by birth and marriage, thank you for encouraging my curiosity, and for always teaching me the value of knowledge. To all of my mentors (Dan, Julie, and Nancy) and colleagues, past and present, thank you for the help getting started in this exciting life.

Chapter 1: Introduction

ADENOVIRAL VACCINE VECTORS

Adenoviruses

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses that replicate in the nucleus of infected cells and do not integrate their DNA into the chromosomes of the infected host cell (1). The adenovirus family consists of multiple distinct genotypes. To date, complete genomes for 71 human adenovirus genotypes are recorded in Genbank (genbank.gov; November 2015), and novel human and non-human primate origin adenoviruses are identified with regularity (2-6). Distinct adenovirus genotypes share genomic organization characteristics and, viral genes are broadly divided into “early” and “late genes”. The early genes encode proteins responsible for initiation of viral transcription, viral replication, and host immune modulation, while the late genes consist of the 11 structural proteins required to assemble a viral particle (7). The late genes are predominantly controlled by the major late promoter (MLP) and individual genes are expressed by differential mRNA splicing (8, 9). While genotypes share genomic organization and basic replication characteristics, unique genotypes display differences in primary attachment receptor usage, tissue tropism, and intracellular trafficking patterns (10). Neutralizing antibodies to a given genotype are usually minimally cross-reactive between genotypes, and hence many genotypes are also distinct serotypes (11). The amenability of genetic manipulation of DNA viruses, lack of viral DNA integration, shared genomic architecture between genotypes, distinct biological properties of virus genotypes, and high immunogenicity make adenoviruses ideal platforms for viral vector development.

Development and construction of adenoviral vectors

The standard “vectorization” process involves the deletion of the E1 and E3 early gene regions, which generates approximately 4 kb of genomic space for the insertion of a foreign transgene of interest (12), usually inserted in the location of the deleted E1 region (13-15). Deletion of the E1 region renders the adenovirus replication-incompetent (16). The E3 region encodes immune evasion genes, one of which functions to down-regulate the expression of MHC class I, and this region is dispensable for replication in tissue culture (17, 18). Replication-incompetent Ad vectors were initially explored as delivery systems for gene therapy (19, 20). As gene therapy tools, Ad vectors expressing an exogenous transgene of interest showed promise *in vivo* in both small animal models as well as in non-human primates. However, a major limitation of the technology is the rapid immune-mediated clearance of transduced cells, primarily by CD8⁺ T cells (21-23). This ability to elicit robust transgene-specific immunity is the reason why Ad vectors are now being extensively pursued as candidate vaccine platforms.

The first description of a replication-incompetent Ad vector as a vaccine platform was against pseudorabies virus (14). This vector construct expressed the transgene product under control of the adenovirus MLP with the transgene inserted into the deleted E1 region, but this construct was only modestly immunogenic. Replacement of the MLP transgene promoter with the cytomegalovirus (CMV) promoter (13), while keeping the transgene placement with the genome the same, enhanced transgene expression and substantially improved immunogenicity. This vector layout is the standard vector architecture currently in use (5, 6, 24).

Preclinical and clinical evaluation of adenoviral vaccine vectors

Replication-incompetent recombinant Ad vectors are currently being investigated as candidate vaccines for a number of infectious diseases that are of considerable public health concern,

including HIV-1 (25, 26), hepatitis C virus (6, 27, 28), tuberculosis (29), malaria (30), and Ebola virus (31, 32), amongst others.

In an early phase human trial of a candidate malaria vaccine using an Ad vector prime followed by a poxvirus vector boost, there was a significant delay in parasitaemia when volunteers were challenged with a heterologous malaria strain (30). This delay in morbidity was strongly correlated with the magnitude of the malaria-specific IFN- γ ⁺ CD8⁺ T cell response. In a non-human primate model, Ad vectors expressing Ebola antigens elicited CD8⁺ T cell responses that were strongly correlated with protection from lethal Ebola virus infection (31), and utilization of an Ad-poxvirus prime-boost regimen induced durable CD8⁺ T cell-mediated protection (32). These data demonstrate that Ad vector-elicited T cell responses can be protective in multiple diseases, both in pre-clinical and clinical studies.

Additional human clinical trials have demonstrated that Ad vector-based regimens induce robust immune responses against hepatitis C virus (HCV), *Mycobacterium tuberculosis* (Mtb), and respiratory syncytial virus (RSV) antigens. Two phase I human trials of Ad vectors for HCV, alone or combined with a poxvirus boost, have demonstrated substantial induction of HCV-specific CD4⁺ and CD8⁺ T cell responses (27, 28), and in non-human primates T cell responses are critical for viral control (33, 34). Ad vectors are also being actively investigated in the context of Mtb infection as a boosting vector for Bacille Calmette-Guerin (BCG)-primed humans (29). Such an approach has displayed protective efficacy against pulmonary tuberculosis in multiple animal models (35-37). Moreover, Ad vectors are being developed against RSV, where they have demonstrated good safety profiles and induced robust T cell and antibody responses in a human phase I clinical trial (38). Further studies are required to determine if these vaccine regimens prove efficacious against these diseases, but the immunogenicity data are promising.

Adenovirus vectors have been most extensively studied in the development of an HIV vaccine. In non-human primate challenge studies, adenovirus serotype 26 (Ad26) vector-elicited T cell responses correlated with reduced viral loads and increased survival after infection with a heterologous, pathogenic strain of SIV (39, 40). More recently, Ad26 vector-based immunization regimens have been shown to elicit antibody responses, which can partially protect non-human primates from viral infection upon challenge with a difficult-to-neutralize strain of SIV or SHIV (simian/human immunodeficiency virus chimera) (40-42). Vaccine regimens based on these data have entered human clinical testing (25, 26). To date, some of the most promising pre-clinical HIV vaccine results have involved Ad vector-based vaccine regimens.

Unfortunately, efficacy trials in humans of a candidate adenovirus serotype 5 (Ad5)-based HIV-1 vaccine failed to demonstrate vaccine-induced protection (43-45). The first reported failures were in the related Merck STEP and Phambili trials (43, 45), and the second was HVTN505 (44). Two key factors likely contributed to the failure of these trials: 1) the specific vaccine regimen utilized, and 2) the choice of transgene antigens. Both trials utilized an Ad5-derived vector, and it is now appreciated that a large fraction of the global population has high Ad5-specific neutralizing antibody responses (46). In animal models, high titers of vector-specific antibodies dampen vaccine-elicited immunity (47). This concept is discussed in more detail in a following section. Additionally, the specific vaccine regimens likely also contributed. The STEP and Phambili trials utilized a homologous Ad5 prime-boost regimen, and we now appreciate that homologous prime-boost regimens are less immunogenic, and show reduced efficacy in non-human primate models than heterologous prime-boost regimens (39). The HVTN505 study utilized a DNA prime-Ad5 vector boost regimen, but in non-human primate models this immunization regimen fails to induce immunity that protects animals from viral

challenge with a difficult-to-neutralize virus strain, and only partially protects against an easy-to-neutralize virus strain (48). These data suggest that the immune responses elicited by the HVTN505 vaccine protocol may have been of insufficient magnitude and/or functionality to protect from infection. Finally, the choice of vector insert(s) is critical to the successful development of an HIV vaccine given the huge global genetic diversity of the virus (49), and selection of optimal transgene antigens is an ongoing area of research (50-52). Despite these discouraging setbacks, revised and improved Ad vector-based vaccine strategies continue to show promise as candidate vaccine regimens against HIV as well as an array of other diseases of considerable importance to global health.

Adenoviral vectors as tools for studying fundamental immunology

Replication-incompetent viral vectors are useful tools for investigating immune regulatory pathways due to their unique biology. Viral and bacterial infections either induce brief immune activation and brief antigen expression (acute infections) or prolonged immune activation and prolonged antigen expression (chronic infections) (53, 54). By contrast, replication-incompetent Ad vectors induce transient innate immune activation (55), with serum cytokine levels returning to baseline by day 3 post-immunization. However, transgene expression can be detected *in vivo* for many weeks post-immunization (56-58). Both the degree of innate immune activation and the duration and amount of antigen have strong influences on the resultant CD8⁺ T cell responses (59, 60). Thus, investigating how T cell responses are regulated following Ad vector immunization can provide fundamental insight into how the immune system responds to a unique means of activation.

ADENOVIRAL VECTOR-INDUCED IMMUNE RESPONSES: T CELLS

CD4⁺ T cell responses

CD4⁺ T cells recognize peptide antigen presented in the context of MHC class II, and were initially described as “T helper” cells due to their ability to provide critical positive signals that orchestrate effector functions of other innate and adaptive cell types (61). One of the key functions of CD4⁺ T cells is to provide positive helper signals to CD8⁺ T cells and B cells. Mechanisms of how CD4⁺ T cells provide help to CD8⁺ T cells is discussed below, and the mechanisms of CD4⁺ T cell help to B cells is discussed in the section on antibody responses. Data presented in this thesis demonstrate that help to CD8⁺ T cells and B cells by CD4⁺ T cells is critical following Ad vector immunization. The current understanding of Ad vector-elicited CD4⁺ T cell responses is reviewed below, with a specific focus on gaps in knowledge.

CD4⁺ T cells are a highly plastic cell population, which is capable of differentiating into multiple subsets expressing different transcriptional programs, and producing a diverse array of cytokines depending on the stimulating environment (62). Historically, several subsets of T helper (T_H) cells have been identified, including: T_H1 cells that provide antiviral immunity, T_H2 cells that are involved in parasite immunity and allergy, T_H17 cells that are involved in inflammatory responses and autoimmune diseases, T_{FH} cells that are specialized to provide help to B cells, and T_{regs} that are a suppressor cell population and limit self-reactive immune responses (63-67). Additional T_H cell subsets with new functions are being identified with some regularity (68-71). However, studies have demonstrated that *in vivo* these differentiation states are not terminal, and that CD4⁺ T cells can interconvert between these states depending on the stimulus, or can express characteristics of multiple T_H lineages simultaneously (72, 73). CD4⁺ T cells display a complex biology, which can even be highly immunopathogenic (74). Thus, a

more complete understanding of their regulation and function can enhance rational vaccine design.

Despite their critical role in regulating innate and adaptive immune responses, relatively little is known about Ad vector-elicited CD4⁺ T cell responses. It has been demonstrated that following intramuscular immunization of mice and rhesus monkeys, Ad vectors induce robust poly-functional T_H1 type CD4⁺ T cell responses, as characterized by the production of IFN- γ , TNF- α , and IL-2 (39, 47, 75). In a mouse model of *Leishmania major* infection, the frequency of these poly-functional vaccine-elicited CD4⁺ T cells was inversely correlated with lesion size (75), which demonstrates the protective capacity of Ad vector-elicited CD4⁺ T cells in pertinent disease models. Thus, Ad vector vaccination can elicit functional T_H1 CD4⁺ T cell responses.

Ad vectors can also elicit CD4⁺ T cell responses that appear to exhibit an immune-modulatory phenotype. It has been reported that increasing the immunizing dose of an Ad5 vector from 10⁷ vp to 10¹⁰ vp actually decreases the T_H1 poly-functionality of the resultant CD4⁺ T cell response, and instead induces a CD4⁺ T cell population that produces large amounts of the immunosuppressive cytokine IL-10 (76). Additionally, the high-dose vaccine was actually less effective than the low-dose vaccine at eliciting protection from *L. major* infection, although this effect was not attributable to IL-10 production. Mechanistically, the signals that regulate the induction of poly-functional CD4⁺ T cell responses versus IL-10-producing less-functional CD4⁺ T cell responses following Ad vector immunization remain to be determined. Furthermore, whether decreased Ad vector vaccine efficacy with increased vaccine dose is a generalizable characteristic of Ad vectors, or a unique trait of Ad5-derived vectors, remains to be determined. Substantial additional work is required to more completely understand the induction of T_H1-type CD4⁺ T cell responses by Ad vector vaccination.

As discussed previously, Ad vector vaccination can also induce transgene-specific antibody responses (38, 41). The induction of antibody responses to the majority of antigens requires helper signals from $CD4^+$ T_{FH} cells (77). It has been previously demonstrated that the absence of $CD4^+$ T cells impairs the development of Ad vector-specific neutralizing antibodies (22, 78). Given the requirement for $CD4^+$ T cells in the induction of antibody responses it is logical to conclude that Ad vectors must induce T_{FH} $CD4^+$ T cells. However, to date, no studies have directly investigated the induction of T_{FH} $CD4^+$ T cells by Ad vector vaccination. The role of $CD4^+$ T cell subsets in the regulation of antibody responses by Ad vector vaccination remains to be explored. In chapter 4 of this thesis I will discuss my findings with regards to $CD4^+$ T cells and the regulation of Ad vector-induced antibody responses.

CD8⁺ T cell responses

Substantial work using prototypic intracellular pathogens has generated a rather complete model of how $CD8^+$ T cell responses are primed and maintained following acute infection. However, given the unique biology of replication-incompetent Ad vectors, it is important to determine how accurately these models describe the processes that regulate Ad vector-elicited $CD8^+$ T cell responses. This section will review the current knowledge of the regulation of Ad vector-elicited $CD8^+$ T cell responses with a specific emphasis on current gaps in knowledge. Areas where this thesis provides novel insights are noted.

Following acute infection or immunization, $CD8^+$ T cells undergo extensive proliferation and differentiation into several heterogeneous subsets of effector and memory cells (79). Complete priming of a $CD8^+$ T cell requires three signals: 1) T cell receptor (TCR) engagement with cognate peptide antigen presented in the context of MHC class I, 2) ligation of co-

stimulatory receptors, and 3) provision of stimulatory cytokines (80). These three signals can simultaneously impact proliferation of responding CD8⁺ T cells, their phenotype, and function (81-85). Thus, when investigating the pathways that regulate Ad vector-elicited CD8⁺ T cell responses, the impact of a given pathway on both accumulation of antigen-specific cells and their phenotype/function must be considered.

In mice, effector and memory CD8⁺ T cells can be differentiated through the expression of the cell surface proteins KLRG1 and CD127 (IL-7R α). KLRG1^{hi}CD127^{lo} expressing cells are termed “terminal effector” and exhibit strong cytotoxic potential, but minimal secondary proliferative potential. KLRG1^{lo}CD127^{hi} expressing cells are “memory precursors” that are capable of long-term survival (59, 79, 86-88). Memory precursor cells can be further subdivided into CD62L⁻CCR7⁻ effector memory (T_{EM}) cells, which have the ability to enter non-lymphoid tissues but have minimal secondary proliferative capacity, and CD62L⁺CCR7⁺ central memory (T_{CM}) cells, which localize within lymphoid tissues and have the greatest proliferative capacity upon secondary antigen exposure (89). Differentiation into effector or memory precursor cells is regulated by the interaction of multiple transcription factors, such as T-box family proteins, Foxo family proteins, Batf, and TCF-1, and transcriptional repressors, such as Id family proteins and Blimp-1 (90-98). Recent analyses using multi-dimensional cytometry by time-of-flight (CyTOF) has demonstrated that subdividing CD8⁺ T cells into just these few cell states is an oversimplification, and that in reality a huge number of unique T cell subsets can be identified (99). However, the simplified segregation of CD8⁺ T cells into effector (KLRG1^{hi}CD127^{lo}), effector memory (CD127^{hi}CD62L⁺), and central memory (CD127^{hi}CD62L⁺) remains experimentally useful.

In experimental models of acute viral infection, upon antigen exposure the majority of CD8⁺ T cells differentiate into highly cytotoxic effector cells with a KLRG1^{hi}CD127^{lo} phenotype. However, upon antigen clearance, KLRG1^{hi} CD8⁺ T cells die and the CD127^{hi} CD8⁺ T cells preferentially survive (88). Over time these surviving cells upregulate CD62L expression, and this coincides with the ability of these cells to produce IL-2 and thereby become fully-functional T_{CM} cells (89). However, following Ad vector immunization a substantial portion of Ag-specific CD8⁺ T cells do not upregulate CD127 (100-103), and this defect in CD127 upregulation is especially pronounced in Ad5-induced responses compared to other serotype vectors (Ad26, Ad28, and Ad35). Regardless of vector serotype used, upregulation of CD62L and capacity to produce IL-2 are relatively low compared to what is observed following acute viral infection (57, 100-103). Given these unusual phenotypic and functional characteristics, Ad vector-elicited CD8⁺ T cell responses represent ideal tools for the identification of novel mechanisms of immune regulation. The pathways that regulate differentiation of Ad vector-elicited CD8⁺ T cells into functional effector cells are unknown, and the mechanisms underlying the atypical memory differentiation are only beginning to be elucidated (discussed below). The data presented in chapters 2 and 3 of this thesis provide compelling evidence that CD4⁺ T cells are critical regulators of CD8⁺ T cell effector differentiation.

Data from several studies have begun to unravel the mechanisms underlying the non-canonical memory characteristics of Ad vector-elicited CD8⁺ T cell responses. Ad vector induced type I interferons are necessary for promoting memory differentiation of CD8⁺ T cells and for the acquisition of increased poly-functional cytokine production capacity (103), and the lack of type I interferons induced by Ad5 (55, 103) likely partially explains the severe impairment in poly-functional CD8⁺ T cell responses elicited by this vector. Prolonged transgene

expression impairs differentiation of Ad5-induced CD8⁺ T cell responses towards a CD127^{hi}CD62L⁺ phenotype (57). Additional work is required to more completely identify the pathways that regulate the phenotype and function of Ad vector-elicited CD8⁺ T cell responses.

The induction of CD8⁺ T cell responses requires priming by CD11c⁺ dendritic cells (DCs) (104), and the coordinated action of multiple subsets of these professional antigen presenting cells (APCs) is required for optimal priming (105, 106). Consistent with this, following Ad vector immunization, CD11c⁺ DCs present antigen, and only this subset induces proliferation of naïve CD8⁺ T cells *in vitro* (107). In this study, the CD8⁺ lymphoid DC subset was the most efficient at inducing CD8⁺ T cell proliferation *in vitro*. *Batf3*^{-/-} mice lack the CD8⁺ CD11c⁺ subset (108), and these mice have severely impaired CD8⁺ T cell responses following Ad vector immunization (109, 110). CD8⁺ DCs have the greatest ability to present extracellular antigens in the context of MHC class I (termed cross-presentation) (108), and the importance of this DC subset in priming Ad vector-elicited CD8⁺ T cell responses suggests that direct transduction of dendritic cells *in vivo* by Ad vectors is not the major mechanism of T cell priming. Collectively, these studies suggest that cross-presentation of Ad vector-derived transgene products by CD8⁺ DCs appears to be a critical for priming CD8⁺ T cell responses.

Intriguingly, one report has demonstrated that transgene-specific CD8⁺ T cell responses can develop following Ad vector immunization of mice that lack MHC class I expression on hematopoietic origin cells (111). In this study, B6 mice were irradiated, reconstituted with K^{b/-} or K^{b/-}D^{b/-} bone marrow, and immunized with an Ad5 vector expressing SIINFEKL [K^b-restricted epitope of chicken ovalbumin (OVA)] or GP33 [D^b-restricted epitope of lymphocytic choriomenengitis virus (LCMV)]. The resultant antigen-specific CD8⁺ T cell responses exhibited delayed expansion and a reduced effector function and phenotype, but CD8⁺ T cell responses

were functional enough to control SIINFEKL-bearing tumors or LCMV infection significantly better than unimmunized controls. Using a “tetracycline-OFF” vector, it has also been reported that prolonged transgene-expression for between 30 and 60 days is required for the normal accumulation and differentiation of Ad vector-induced CD8⁺ T cell responses (57). Silencing of transgene expression prior to day 30 significantly reduced the magnitude of the Ag-specific CD8⁺ T cell response, and the responding cells produced less of the effector cytokine TNF- α and expressed a more memory phenotype with elevated expression of CD127 and CD62L. Together, these studies suggest a model where CD8⁺ DCs are the primary cells for priming robust Ad vector-elicited CD8⁺ T cell responses, but that long-term antigen presentation on non-hematopoietic cells also plays an important role in the maintenance and effector differentiation of these responses.

In addition to TCR engagement CD8⁺ T cells require supplemental signals through co-stimulatory receptors (signal 2) (112). These receptors are comprised of immunoglobulin (Ig) superfamily proteins, such as CD28, and tumor necrosis factor receptors (TNFR) superfamily proteins, such as CD70, OX-40, and 4-1BB. All of these receptors bind to ligands that are membrane-bound proteins, and thus direct cell-cell contact is required for signal 2 to be transmitted to T cells. The most well-studied co-stimulatory signaling pathway is CD80/CD86 expressed on APCs interacting with CD28 expressed on T cells (113). CD80 and CD86 double-knockout mice immunized with Ad vectors have undetectable Ag-specific CD8⁺ T cells at the time of peak-immunogenicity (110). Absence of CD28 also dramatically impairs Ag-specific CD8⁺ T cell responses, but not to the equivalent degree as in the CD80/CD86 deficient mice (110). Intriguingly, in CD28 deficient mice, T cell responses of equal magnitude to those observed in wild type animals ultimately developed by day 60 post-immunization, but these cells

exhibit long-term functional defects in cytokine production, anamnestic potential, and the ability to control viral infections. Thus, signals via the CD80/CD86 pathway are critical for the accumulation and development of functional Ad vector-elicited CD8⁺ T cells.

Signaling via TNFR family proteins is also important for the development of functional Ad vector-elicited CD8⁺ T cell responses, but the data are less conclusive. Blockade of CD70 (the ligand for CD27) prior to intravenous Ad vector immunization reduces the magnitude of Ag-specific CD8⁺ T cell responses by ~50% (114). However, CD8⁺ T cell responses were only assessed at a single time point post-immunization and functional analysis was not performed. Furthermore, it remains to be determined if CD8⁺ T cell responses elicited by intravenous versus the standard intramuscular immunization regimen are regulated equivalently. One study found no perturbation in the frequency of Ag-specific CD8⁺ T cells following Ad vector immunization of OX-40 KO mice, with no assessment of phenotype or functionality (110). However, an earlier study found that specific absence of OX-40 expression on CD8⁺ T cells results in decreased proliferation, cytotoxic capacity, cytokine production, and anamnestic potential (115). The reason for these discrepancies remains to be determined. This same study also determined that cell-intrinsic signaling via 4-1BB on CD8⁺ T cells actually restrains responses following Ad vector immunization, as deletion of 4-1BB increases the magnitude of the response, cytokine and cytotoxic capacity, and increases anamnestic potential (115). Thus, TNF family receptors appear to play critical roles in modulating Ad vector-elicited CD8⁺ T cell responses, but substantial further work is required in this area to clarify the role of this family of receptors.

The final set of signals required for optimal priming of CD8⁺ T cells are stimulatory cytokines (signal 3). The first described and most extensively studied of these signal 3 cytokines are IL-12 and type I interferons (116-118). More recently, IL-21 has also been shown to have

“signal 3” properties (119). With regards to Ad vector immunization, type I interferons have an anti-proliferative effect on Ad28- and Ad35-induced CD8⁺ T cell responses when the dose of vector is low (5×10^7 viral particles), but have no impact on CD8⁺ T cell responses following the standard 1×10^9 viral particle dose (103). Absence of type I interferon signaling has no impact on CD8⁺ T cell responses induced by low or high dose Ad5 immunization. A role for IL-12 in modulating Ad vector-elicited responses has not been directly tested, but serological analysis of rhesus monkeys immunized with several different serotype Ad vectors observed no IL-12 induction (55). Although not conclusive, these data suggest that IL-12 does not play a major role in regulating CD8⁺ T cell responses following Ad vector vaccination. IL-21R KO mice exhibit modest defects in the frequency of antigen-specific CD8⁺ T cells following primary immunization and these cells exhibit major defects in anamnestic potential, but no perturbation in primary phenotype or cytokine producing potential are observed (120). Thus, of the three well-characterized signal 3 cytokines, IL-21 appears the most important in the context of Ad vector vaccination, but even this cytokine appears to have only a minor role in regulating the frequency of Ad vector-elicited CD8⁺ T cells. Thus, more work is required to understand the pathways that regulate the proliferation CD8 T cell responses induced by Ad vector immunization. In chapter 2 and 3, I will present data that CD4⁺ T cells regulate CD8⁺ T cell proliferation, and this help may replace the need for signal 3 cytokines for these responses.

In experimental systems other than Ad vector immunization, differentiation to cytotoxic and cytokine producing effector cells does not occur when signal 3 cytokine signaling is inhibited. However, with regards to Ad vector immunization, no studies have comprehensively defined the signals that regulate effector function of Ad vector-elicited CD8⁺ T cell responses. In this thesis I will present compelling data that CD4⁺ T cell-derived signals are critical for CD8⁺ T

cell effector differentiation following Ad vector vaccination, and that CD4⁺ T cells are key regulators of vaccine-induced CD8⁺ T cell responses. A summary of the literature with regards to CD4⁺ T cell help to CD8⁺ T cells follows.

CD4⁺ T cell help to CD8⁺ T cell responses

A key regulator of CD8⁺ T cell responses is CD4⁺ T cells, as their absence leads to proliferative and functional defects (121-130). Interestingly, the importance of CD4⁺ T cell help and the pathways involved varies greatly based on the specific experimental system (131). In several well-studied infection models CD8⁺ T cells display unimpaired expansion in the absence of CD4⁺ T cells (132-134). In contrast, CD4⁺ T cell help mediated by CD40 signaling is required for the development of primary CD8⁺ T cell responses following exposure to antigens that induce weak innate inflammatory responses (122, 135, 136). CD4⁺ T cells also play a critical role in reducing CD8⁺ T cell exhaustion following chronic infection (137, 138). Thus, while CD4⁺ T cell help is critical in many settings to promote and maintain functional CD8⁺ T cell responses, this help can be highly context dependent. The helper function of CD4⁺ T cells following immunization with viral vector vaccines remains poorly defined.

The optimal expansion of an Ad vector-elicited primary CD8⁺ T cell response requires CD4⁺ T cell help. CD4⁺ T cell help is required for induction of transgene-specific CD8⁺ T cell responses (139, 140). Viral vector-specific CD8⁺ T cell responses also develop following immunization, and these responses are sufficient to clear transduced cells (141). The clearance of transduced cells requires CD4⁺ T cells (21), and therefore, it appears that vector-specific CD8⁺ T cell responses also require CD4⁺ T cell help. Given the requirement for CD4⁺ T cell help for primary expansion of CD8⁺ T cell responses, Ad vectors appear to behave like other “less

inflammatory” infection/immunization systems (mentioned above). Consistent with this, antagonism of the CD40 signaling pathway (by antibody or gene knockout) impairs the development of vector-specific CD8⁺ T cell responses (136, 142-144). As absence of CD4⁺ T cells and absence of signaling via the CD40 pathway induce the same phenotype, and as ligation of CD40 can replace the need for CD4⁺ T cells in other systems (128-130), it is presumed that this also reflects a mechanism of CD4⁺ T cell help in the context of Ad vector immunization. In summary, CD4⁺ T cell help is required for the robust induction of CD8⁺ T cell responses by Ad vector vaccination.

Despite a clear role for CD4⁺ T cells in regulating Ad vector-elicited CD8⁺ T cell responses, many unknowns remain. In other low-inflammation experimental systems where CD4⁺ T cell help is required for priming of CD8⁺ T cell responses, CD4⁺ T cell-derived IL-2 has also been implicated as a critical mechanism (122). The role of IL-2 in regulating Ad vector-elicited CD8⁺ T cell responses has not been assessed. While CD40L expression and production of IL-2 by CD4⁺ T cells are the two best described mechanisms of CD4⁺ T cell help, CD4⁺ T cells can produce a large array of cytokines and express several other cell surface ligands that can induce DC maturation (145-147). IL-21, a cytokine produced by CD4⁺ T cells, has been shown to be important in maintaining CD8⁺ T cell frequency and functionality during chronic viral infection (148-150), and absence of IL-21 impairs primary frequency and anamnestic potential of Ad vector-elicited CD8⁺ T cell responses (120), as mentioned above. However, the impact on the frequency of primary CD8⁺ T cell responses is modest compared to the major defects seen in the absence of CD4⁺ T cell, and therefore suggests other CD4⁺ T cell-derived signals are also required. If consideration of other CD4⁺ T cell-derived cytokines is restricted to just the canonical T_H1 cytokines of IFN- γ , TNF- α , and IL-2, then a paucity of data exists

regarding the role of CD4⁺ T cell-derived IFN- γ and TNF- α in regulating CD8⁺ T cell responses, despite strong data that these two cytokines have important impacts on CD8⁺ T cell function (151, 152). Furthermore, a role for CD4⁺ T cells in regulating CD8⁺ T cell effector differentiation following priming with a low-inflammation immunization/infection, such as Ad vectors, has not been described. Following LCMV infection, CD4⁺ T cells restrain effector differentiation (124), but if such a role exists for CD4⁺ T cells in the context of Ad vector immunization remains unknown. Despite the initial direct *in vivo* observation that CD4⁺ T cells can regulate CD8⁺ T cell responses being made over 30 years ago (153), our understanding of how CD4⁺ T cells provide help to CD8⁺ T cells remains incomplete. The data presented in this thesis will substantially enhance our understanding of how CD4⁺ T cells promote primary Ad vector-elicited CD8⁺ T cell responses, and will for the first time demonstrate a critical role for CD4⁺ T cells in the acquisition of cytotoxic effector function by CD8⁺ T cells.

ADENOVIRAL VECTOR-INDUCED IMMUNE RESPONSES: ANTIBODIES

There is growing interest in the use of Ad vectors as vaccine platforms for the induction of antibody responses. However, an understanding of the processes that control these responses is lacking. This section will review the current knowledge of antibodies elicited by Ad vector immunization, and identify areas where the data presented in this thesis provide novel insights.

Fundamentals of Ad vector-elicited antibody responses

Ad vectors elicit two types of antibody responses: 1) transgene-specific antibodies, and 2) vector-specific neutralizing antibodies. In the context of HIV vaccine development it has recently been demonstrated that Ad vectors can elicit robust transgene-specific antibody

responses (40-42). In these studies, Ad vector prime followed by either heterologous Ad vector boost, poxvirus boost, or recombinant protein boost induces antibody responses that were capable of partially protecting non-human primates from a heterologous difficult-to-neutralize SIV or SHIV (simian/human immunodeficiency virus) viral challenge. Additionally, a recent phase I trial of a candidate Ad vector-based respiratory syncytial virus (RSV) vaccine elicited robust RSV-specific antibody responses in human volunteers (38). However, data is lacking with regards to fundamental knowledge of how Ad vector-elicited transgene-specific antibody responses are regulated, and substantial work is required in this area.

In contrast to the scarce data regarding transgene-specific antibodies, there has been more research into understanding vector-specific antibody responses. Vector-specific neutralizing antibodies are a major impediment to effective use of Ad vectors as gene therapy or vaccine platforms. Pre-existing vector-specific immunity dramatically reduces the efficiency of transgene delivery upon vector administration (22), and thereby reduces the potency of vaccine-elicited T cell responses (154-156). Vector-specific neutralizing antibodies can be present in an individual due to either pre-existing immunity by prior natural exposure to a given serotype adenovirus, or by immunization with an Ad vector of a given serotype. As a cogent example, in a phase IIb/III clinical trial of a candidate Ad serotype 5 (Ad5) vector-based HIV vaccine, high titer baseline Ad5-specific immunity was found to significantly reduce the immunogenicity of the candidate vaccine (43). Thus, vector-specific immunity is a major technical hurdle for the utilization of Ad vectors as vaccine platforms.

To circumvent the issue of pre-existing immunity two related strategies have been employed: 1) development of novel Ad vectors derived from adenoviruses with low sero-prevalence, and 2) modification of viral vectors of high sero-prevalence origin to express

neutralization determinants of low sero-prevalence virus types. In all three phase IIb/III HIV-I clinical trials utilizing Ad vectors, Ad5 vectors were utilized (43-45), and Ad5 vectors have been the most extensively studied. However, sero-prevalence analysis from sub-Saharan Africa has identified Ad5-specific neutralizing antibody titers of >1000 in between 43% and 50% of individuals (5, 15, 24). Thus, alternative serotype Ad vectors, of both human and non-human primate origin, have been developed (5, 6, 15, 24). These vectors display virus-specific titers >1000 in 35% of individuals for the most sero-prevalent vector, Ad28, down to 0% of individuals for multiple of the non-human primate-derived vectors. Most importantly, these alternate serotype vectors exhibit no reduction in immunogenicity in animals with high titers of Ad5-specific neutralizing antibodies (15, 157). Thus, development of novel, alternate serotype Ad vectors represents a promising avenue to circumvent pre-existing immunity.

An alternate approach is to modify the Ad5-derived vector to circumvent pre-existing immunity. The major neutralization determinant of adenovirus vectors is the seven hyper-variable regions (HVR) (1-7) of the hexon surface protein of the virus capsid (158, 159). A chimeric Ad5HVR48(1-7) virus vector, where the Ad5 HVRs are replaced with the HVRs from the serologically distinct Ad48 virus, bypasses pre-existing Ad5-specific neutralizing antibodies. This chimeric vector can elicit robust immune responses in mice and non-human primates with pre-existing Ad5 immunity (159). However, a potential limitation of such an approach is that only specific backbone-HVR chimeras result in viable viral vectors (160). Thus, Ad vectors can be specifically engineered to reduce issues of pre-existing vector-specific immunity.

In summary, Ad vector vaccination can elicit both desirable transgene-specific antibody responses, the goal of vaccination, and undesirable vector-specific neutralizing antibody

responses, which can reduce vaccine immunogenicity and prevent the use of homologous prime-boost regimens.

Regulation of Ad vector-elicited antibody responses by CD4⁺ T cells

Understanding how Ad vector-elicited transgene-specific and vector-specific antibody responses may provide novel insights into antibody biology, and thereby improve vaccine design. As induction of transgene-specific antibody responses are the goal of vaccination, insights into the regulation of these responses is of particular interest. Unfortunately, the regulation of these responses remains poorly described, and studies in this area are sorely needed.

Thankfully, there are substantial data on how vector-specific antibody responses are regulated. One of the key regulators of antibody responses are CD4⁺ T cells, and absence of these cells at the time of antigen-exposure results in tolerance towards the antigen (161-163). The development of an antibody response is a complex multi-step process that involves the formation of a B cell germinal center, a dynamic physical structure within the secondary lymphoid tissue (164). The germinal center is where B cells undergo affinity maturation by AID-mediated somatic hyper-mutation and class-switch recombination (165, 166). The formation and maintenance of germinal centers is absolutely dependent on CD4⁺ T_{FH} cells (64). Consistent with this model, the development of vector-specific neutralizing antibodies is dependent on the presence of CD4⁺ T cells (22, 78). T_{FH} cells provide help to B cells by cell surface bound ligand-receptor interactions, as well as through soluble cytokines (77). One of the major signaling pathways in this process is ligation of CD40 expressed on B cells by CD40L expressed on CD4⁺ T cells (167, 168). The CD40 signaling pathway is required for the development of vector-specific antibody responses upon Ad vector administration (136, 144, 169, 170). However, as

CD4⁺ T cell regulation of antibody responses requires many more signals than just CD40, substantial work remains to generate a complete picture of the pathways involved in the induction of vector-specific antibody immunity.

Despite growing interest in the use of Ad vectors as priming platforms for the induction of vaccine-induced antibody responses, relatively little is known regarding the regulation of these responses. With respect to vector-specific neutralizing antibodies, prior studies suggest that the same processes described in other experimental systems regulate these responses. However, the degree to which this is accurate with regards to specific signaling pathways remains to be determined. Furthermore, extensive work is required to understand, even in the broadest terms, how transgene-specific antibody responses are regulated. Chapter 4 of this thesis describes the role of CD4⁺ T cells in regulating Ad vector-induced transgene-specific antibody responses, and by doing so identifies novel parameters for CD4⁺ T cell regulation of antibody responses.

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**Chapter 2: Longitudinal Requirement for CD4⁺ T Cell Help for Adenovirus Vector-
Elicited CD8⁺ T Cell Responses**

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ABSTRACT

Despite the widespread use of replication-incompetent recombinant adenovirus (Ad) vectors as candidate vaccine platforms, the mechanism by which these vectors elicit CD8⁺ T cell responses remains poorly understood. Our data demonstrate that induction and maintenance of CD8⁺ T cell responses by Ad vector immunization is longitudinally dependent on CD4⁺ T cell help for a prolonged period of time. Depletion of CD4⁺ T cells in wild type mice within the first eight days following Ad immunization resulted in dramatically reduced induction of antigen-specific CD8⁺ T cells, decreased T-bet and Eomesodermin expression, impaired KLRG1⁺ effector differentiation, and atypical expression of the memory markers CD127, CD27, and CD62L. Moreover, these CD8⁺ T cells failed to protect against a lethal recombinant *Listeria monocytogenes* challenge. Depletion of CD4⁺ T cells between week 1 and week 4 following immunization resulted in increased contraction of memory CD8⁺ T cells. These data demonstrate a prolonged temporal requirement for CD4⁺ T cell help for vaccine-elicited CD8⁺ T cell responses in mice. These findings have important implications in the design of vaccines aimed at eliciting CD8⁺ T cell responses and may provide insight into the impaired immunogenicity of vaccines in the context of AIDS and other CD4⁺ T cell immune deficiencies.

INTRODUCTION

Adenovirus vectors have garnered significant attention as candidate vaccine platforms due to their large transgene coding capacity and potent immunogenicity. Ad vector-based vaccines are being pursued for a number of viral infections, including ebola (1), influenza (2), hepatitis C (3, 4), rabies (5) and HIV-1 (6). Recently we have evaluated an Ad26 vector-based vaccine for HIV-1 in clinical trials (7, 8), and preclinical studies with Ad26-based vaccine regimens in non-human primates resulted in partial protection against acquisition of infection as well as virologic control following SIV_{mac251} challenges (9-11). Virologic control correlated with vaccine-elicited SIV-specific CD8⁺ T cell responses (9-12). However, relatively little is known about the CD4⁺ T cell requirement to generate CD8⁺ T cell memory responses following vaccination.

Prior reports have evaluated Ad vectors as candidate vaccine and gene therapy platforms and have identified a role for CD8⁺ T cells in the clearance of transduced cells (13). Several follow-up studies have demonstrated prolonged transgene expression in the absence of CD4⁺ T cells at the time of vector administration, thus providing evidence that CD4⁺ T cells play an important role in priming the CD8⁺ T cell response following Ad vector administration (13-16). More recent studies have demonstrated that the frequency of antigen-specific CD8⁺ T cells was impaired in the absence of CD4⁺ T cells at the time of vector administration (17, 18). Lack of CD4⁺ T cells also resulted in primary CD8⁺ T cell responses of low magnitude and function in several disease and vaccination models (19-22). In contrast, in certain viral and bacterial infections, CD8⁺ T cell responses were induced in the absence of CD4⁺ T cells, although the long-term functional potential and maintenance were still impaired (23-30). CD4⁺ T cell help has also been reported to be required at the time of priming to elicit CD8⁺ T cell responses with normal recall potential upon secondary antigen exposure (31-33). These studies show the

requirement of CD4⁺ T cell help at the time of CD8⁺ T cell priming, but the temporal requirements of CD4⁺ T cell help for the generation of CD8⁺ T cell responses have not previously been determined.

In the present study, we sought to determine the temporal requirements of CD4⁺ T cell help for the development, maintenance and functionality of memory CD8⁺ T cells induced by Ad26 (34) and Ad5HVR48 (35) vectors expressing SIV Gag, SIV Env, and LCMV GP antigens. We selected Ad26 and Ad5HVR48 vectors for detailed study as they are both currently being evaluated in phase I clinical trials as vaccine candidates. We found that CD4⁺ T cell help was required not only at the time of priming but also for eight days post-immunization to drive the induction and optimal effector differentiation of the primary CD8⁺ T cell response. Moreover, CD4⁺ T cell help was required for four weeks post-immunization for controlling the contraction of memory CD8⁺ T cells.

MATERIALS AND METHODS

Mice, immunizations, and challenge. Six- to ten-week-old C57BL/6, B6.SJL-ptprc^a (CD45.1⁺), B6.129S2-Cd4^{tm1Mak}/J (CD4 KO), B6.129S2-H2^{dlAb1-Ea}/J (MHC II KO), B6.129S2-Cd40lg^{tm1Imx}/J (CD40L KO), and B6.129P2-Cd40^{tm1Kik}/J (CD40 KO) animals were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were immunized with the previously described E1/E3 deleted Ad26, or Ad5HVR48(1-7) vectors expressing SIV Gag or SIV Env from the strain SIV_{mac239}, or LCMV GP (11, 34-36). Mice were immunized i.m. in the quadriceps with 10⁹ viral particles of each vector in a volume of 100µl divided equally between the two legs. For co-administration of SIV Gag and SIV Env expressing vectors the final injection volume of 100µl was held constant. Mice were challenged with 1.75x10⁵ to 2.5x10⁵ cfu of recombinant *Listeria monocytogenes* expressing the LCMV epitope GP33-41 (Lm-GP33) by i.v. injection (a kind gift of Dr. Hao Shen)(37). Precise dose was back calculated following each experiment by plating on BHI-Agar plates, as previously described (38). All animal experiments were in accordance with Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee guidelines.

Monoclonal antibody administration. The monoclonal anti-CD4 antibody GK1.5 (BioXcell) was administered by two i.p. injections of 500µg in a 500µl volume on sequential days. One day after the second injection, blood was collected and CD4⁺ T cell depletion was confirmed (data not shown) by staining with the non-competing anti-CD4 antibody clone RM4-4 (BD Biosciences).

Tissue processing and flow cytometry. Single cell suspensions of tissues were generated as previously described, with slight modification (39). Briefly, liver tissue was not treated with EDTA or collagenase. Peripheral blood mononuclear cells (PBMC) were isolated from whole

blood by Ficoll-Hypaque density centrifugation at 1900 rpm for 20 min. MHC class I tetramer staining was performed using an H-2D^b tetramer loaded with the immunodominant AL11 peptide (AAVKNWMTQTL) or GP33-41 peptide (KAVYNFATM) as previously described (40). Biotinylated class I monomers were kindly provided by the NIH Tetramer Core Facility (Emory University, GA). Background staining of cells from naïve animals was ≤0.1%. Surface staining was performed with anti-CD8α (53-6.7), -CD4 (RM4-5), -CD44 (IM7), -CD127 (A7R34), -CD62L (MEL-14), -KLRG1 (2F1), -CD122 (TM-β1), and -CD27 (LG.3A10). Transcription factor staining was performed by first permeabilizing the cells with the FoxP3 Fixation/Permeabilization kit (eBioscience) and subsequently staining with anti-T-bet (4B10). Annexin V staining was performed using an Annexin V staining kit (BioLegend). All antibodies were purchased from BD Biosciences, eBioscience, or BioLegend. Vital exclusion dye was purchased from Invitrogen. After fixation, samples were acquired on an LSR II flow cytometer (BD Biosciences) and data was analyzed using FlowJo v9.3.3 (Treestar).

Intracellular cytokine staining. Intracellular cytokine staining was performed as previously described with slight modification (41). Briefly, 2x10⁶ splenocytes were re-stimulated for 1.5hrs at 37°C with 2μg/ml of AL11 peptide or 1μg/ml of an overlapping SIV_{mac239} Gag or SIV_{mac239} Env peptide pool. At this time anti-CD28 (37.51), and -CD49d (R1-2) antibodies were added. After this incubation, Brefeldin A (BD Biosciences) was added and samples incubated for an additional 4.5hrs at 37°C. Cells were subsequently washed, stained with surface antibodies, permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with an anti-IFN-γ (XMG1.2) antibody.

CFSE labeling and adoptive cell transfer. Splenocytes were processed as described above. CD8⁺ T cells were enriched by negative selection using the CD8a⁺ enrichment kit II following

the manufacturers instructions (>90% purity) (Miltenyi). CFSE labeling was performed as previously described (42). After labeling, 1.5×10^4 D^b/AL11⁺ CD8⁺ T cells were transferred by i.v. injection into congenic marker differentiated naïve animals. For Figure 8, CFSE labeling was not performed and 5×10^4 D^b/AL11⁺ CD8⁺ T cells were transferred; all other preparation steps were identical.

RNA extraction, cDNA synthesis, and qPCR. Splenocytes were processed as described above. CD8⁺ T cells were enriched by negative selection using the CD8a⁺ enrichment kit II following the manufacturers instructions (>90% purity) (Miltenyi). D^b/AL11⁺ CD8⁺ T cells were subsequently sorted to >95% purity on a FACS Aria (BD Biosciences). Cells were centrifuged at 10,000rpm for 5 minutes and pellets were resuspended in 1ml of TRIzol (Invitrogen) and stored at -80°C for further processing. RNA extraction and cDNA synthesis were performed as previously described (43, 44). Briefly, RNA extraction was performed using the RNeasy Tissue Isolation kit (Qiagen). cDNA synthesis was performed using the Ovation Pico WTA v2 kit (NuGEN) per the manufacturers instructions. Quantitative PCR was performed using the SYBR Green quantification system (QIAGEN). The following primers were used: *Gapdh* forward 5'- AGGTCGGTGTGAACGGATTTG -3' and reverse 5'- TGTAGACCATGTAGTTGAGGTCA -3' (45); *Prdm1* forward 5'- CACACAGGAGAGAAGCCACA-3' and reverse 5'-TTGATTCGGGTCAGATCCTC-3' (46); *Bcl6* forward 5'-CGCAACTCTGAAGAGCCACCTGCG-3' and reverse 5'- TTTGTGACGGAAATGCAGGTTA-3' (47); *Tbx21* forward 5'- AGCAAGGACGGCGAATGTT-3' and reverse 5'-GGGTGGACATATAAGCGGTTC-3' (48); *Eomes* forward 5'-TGAATGAACCTTCCAAGACTCAGA-3' and reverse 5'- GGCTTGAGGCAAAGTGTGACA-3' (49); *Id2* forward 5'-

ACCAGAGACCTGGACAGAAC-3' and reverse 5'-AAGCTCAGAAGGGAATTCAG-3' and *Id3* forward 5'-GACTCTGGGACCCTCTCTC-3' and reverse 5'-ACCCAAGTTCAGTCCTTCTC-3' (50); and *Tcf7* forward 5'-AGCTTTCTCCACTCTACGAACA-3' and reverse 5'-AATCCAGAGAGATCGGGGGTC-3' (51). Data were acquired on a StepOnePlus Real-Time PCR System (Applied Biosystems). Cycle conditions were: 95°C for 10min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Gene expression was normalized across samples by *Gapdh* expression levels.

Statistical analysis. Statistical analysis was performed using a two-tailed non-parametric Mann-Whitney U test or Mantel-Cox test using Prism v6.0c (GraphPad Software Inc). In all cases the CD4⁺ T cell deficient animals or CD40/L knockout animals were compared to the wild type/untreated controls.

RESULTS

CD4⁺ T cell help is required for the induction of CD8⁺ T cell responses following Ad vector immunization

We initially assessed the requirement for CD4⁺ T cell help for the induction of primary CD8⁺ T cell responses following Ad26 vector immunization (34). C57BL/6, CD4 KO, or MHC class II KO mice were immunized intramuscularly with 10⁹ vp of Ad26 expressing SIV Gag (Ad26-Gag). Following immunization, robust Gag-specific CD8⁺ T cell responses were detected in wild type animals by CD8⁺ T cells binding to the immunodominant D^b-restricted AL11 peptide (D^b/AL11) tetramer (40) (Fig. 2.1A and B). This response peaked at day 14 post-immunization and subsequently contracted, consistent with prior observations (34, 40). In contrast, in CD4⁺ T cell deficient animals (CD4 KO or MHC class II KO), the AL11 epitope-specific CD8⁺ T cell responses were largely abrogated ($p < 0.01$; Fig. 2.1A and B).

To confirm that these observations were generalizable to multiple epitopes, C57BL/6, CD4 KO, and MHC class II KO animals were immunized with 10⁹ vp each of Ad26 vectors expressing SIV Gag and SIV Env. Twenty-eight days post-immunization, splenocytes were harvested and IFN- γ production by CD8⁺ T cells was assessed by *ex vivo* re-stimulation with overlapping SIV Gag and SIV Env peptide pools. Gag- and Env-specific responses were detected in wild type animals by percentage of SIV-specific IFN- γ ⁺ CD8⁺ T cells (Fig. 2.1C and D). The frequency of Gag- and Env-specific IFN- γ ⁺ CD8⁺ T cells was significantly impaired in CD4 KO and MHC class II KO mice as compared to wild type animals ($p < 0.01$; Fig. 2.1C and D). These data confirm that the defect seen in CD8⁺ T cell responses in the absence of CD4⁺ T cells by D^b/AL11 tetramer binding was generalizable to multiple antigens.

To confirm that our observations with Ad26-based vectors were generalizable to other Ad vector serotypes, C57BL/6, CD4 KO, and MHC class II KO animals were immunized with 10^9 vp each of Ad5HVR48-Gag and Ad5HVR48-Env (11, 35). Concordant with the data generated following Ad26 immunization, the Gag- and Env-specific $CD8^+$ $IFN-\gamma^+$ T cell responses following Ad5HVR48 immunization were significantly impaired on day 28 post-immunization in CD4 KO and MHC II KO animals as compared to wild type animals ($p < 0.01$; Fig. 2.1E). Collectively, these data demonstrate that the induction of primary $CD8^+$ T cell responses following Ad vector immunization critically requires $CD4^+$ T cell help at the time of priming.

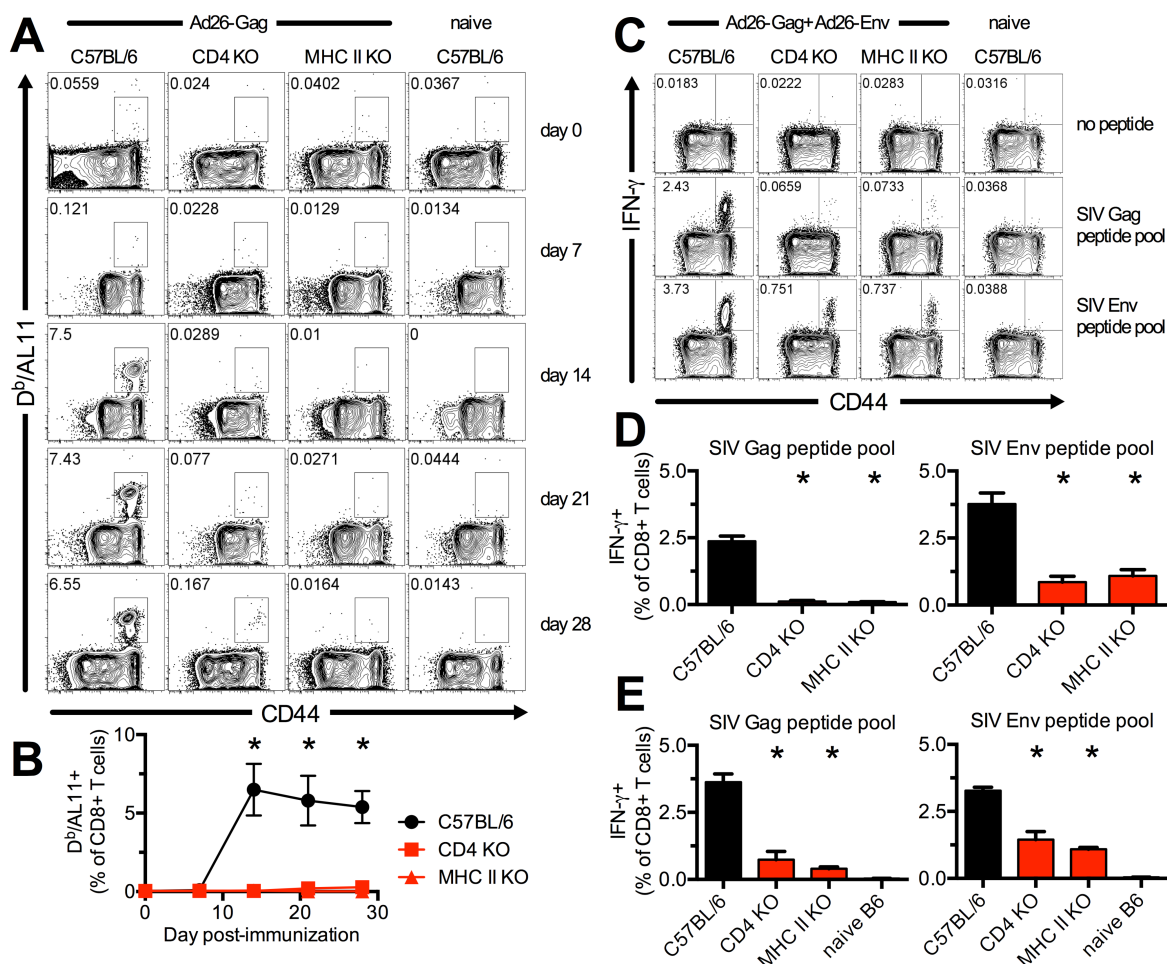


Figure 2.1. CD4⁺ T cell help is required for the development of CD8⁺ T cell responses following intramuscular Ad vaccination. (A and B) C57BL/6, CD4 KO, or MHC class II KO mice were immunized i.m. with 10^9 vp of Ad26-Gag or were unimmunized (naïve C57BL/6). Animals were bled longitudinally and SIV Gag-specific CD8⁺ T cells were quantified by tetramer staining. Representative plots (A) and group averages (B) for $D^b/AL11^+$ cells as a percent of CD8⁺ T cells are shown. Data are from n=8/group (pooled from two independent experiments). (C and D) C57BL/6, CD4 KO, or MHC class II KO mice were co-immunized intramuscularly with 10^9 vp each of Ad26-Gag and Ad26-Env. Splenocytes were harvested on day 28 post-immunization and stimulated with the indicated peptide pool. Representative plots of IFN- γ^+ CD8⁺ T cells (C) and group averages (D) are shown. Data are from n=8/group (pooled from two independent experiments). (E) C57BL/6, CD4 KO, or MHC class II KO mice were co-immunized intramuscularly with 10^9 vp each of Ad5HVR48-Gag and Ad5HVR48-Env. Splenocytes were harvested on day 28 post-immunization and stimulated with the indicated peptide pool. Percent of IFN- γ^+ cells as a fraction of CD8⁺ T cells are shown. Data are from n=8/group (pooled from two independent experiments). Mean \pm SEM are shown; *, P<0.01.

Longitudinal interruption of CD4⁺ T cell help impairs the expansion and increases contraction of CD8⁺ T cell responses

We next sought to determine for how long CD4⁺ T cell help was required post-immunization for the induction of CD8⁺ T cell responses. To accomplish this, CD4⁺ T cells were depleted by administration of the monoclonal anti-CD4 antibody GK1.5 on two consecutive days starting at various time points following immunization (52). Depletion of CD4⁺ T cells was begun prior to immunization (day -1) or on day 1 through 13 post-immunization (Fig. 2.2A). All mice were immunized intramuscularly with 10⁹ vp of Ad26-Gag on day 0, and PBMC were evaluated longitudinally to assess D^b/AL11⁺ CD8⁺ T cell responses.

D^b/AL11⁺ CD8⁺ T cell responses in the untreated group were detected on day 7 (data not shown), peaked on day 14, and contracted by day 28 (Fig. 2.2B, black bar). Depletion of CD4⁺ T cells prior to immunization completely ablated the D^b/AL11⁺ CD8⁺ T cell response (Fig. 2.2B-D), consistent with the data from CD4 KO and MHC II KO animals in the previous experiment (Fig. 2.1A). Administration of an isotype control antibody had no impact on CD4⁺ T cell frequency or the D^b/AL11-specific CD8⁺ T cell response (data not shown). Depletion of CD4⁺ T cells starting on day 1 to day 8 post-immunization resulted in dramatically lower D^b/AL11⁺ CD8⁺ T cell responses from day 14 onwards ($P < 0.05$; Fig. 2.2B and C). Earlier depletion of CD4⁺ T cells led to more pronounced impairment of D^b/AL11⁺ CD8⁺ T cell responses. Mice that were depleted of CD4⁺ T cells on or after day 9 exhibited initial CD8⁺ T cell response magnitudes that were comparable to untreated animals (Fig. 2.2B and C). These data suggest a critical requirement for CD4⁺ T cell help for the induction of CD8⁺ T cell responses for eight days following immunization.

We next examined D^b/AL11⁺ CD8⁺ T cell responses in various tissues. Consistent with the data from peripheral blood, D^b/AL11⁺ CD8⁺ T cell responses in both spleen and liver on day 28 were also reduced in animals depleted of CD4⁺ T cells with similar kinetics (Fig. 2.2D). Moreover, the absolute number of functional AL11-specific IFN- γ ⁺ CD8⁺ splenocytes on day 28 by ICS assays was also reduced in animals depleted of CD4⁺ T cells as compared with untreated controls (Fig. 2.2E).

We next asked whether CD4⁺ T cells were also important during the contraction phase of the CD8⁺ T cell responses. Animals depleted of CD4⁺ T cells beginning on day 9 to day 13 exhibited equivalent initial D^b/AL11⁺ CD8⁺ T cell magnitudes as compared to untreated controls (day 14). However, we observed greater contraction of the Gag-specific CD8⁺ T cell responses ($p < 0.001$; Fig. 2.2B *inset*) in the mice depleted of CD4⁺ T cells on day 9-13 as compared to untreated controls, suggesting a requirement for longitudinal CD4⁺ T cell help for controlling the contraction of CD8⁺ T cell responses.

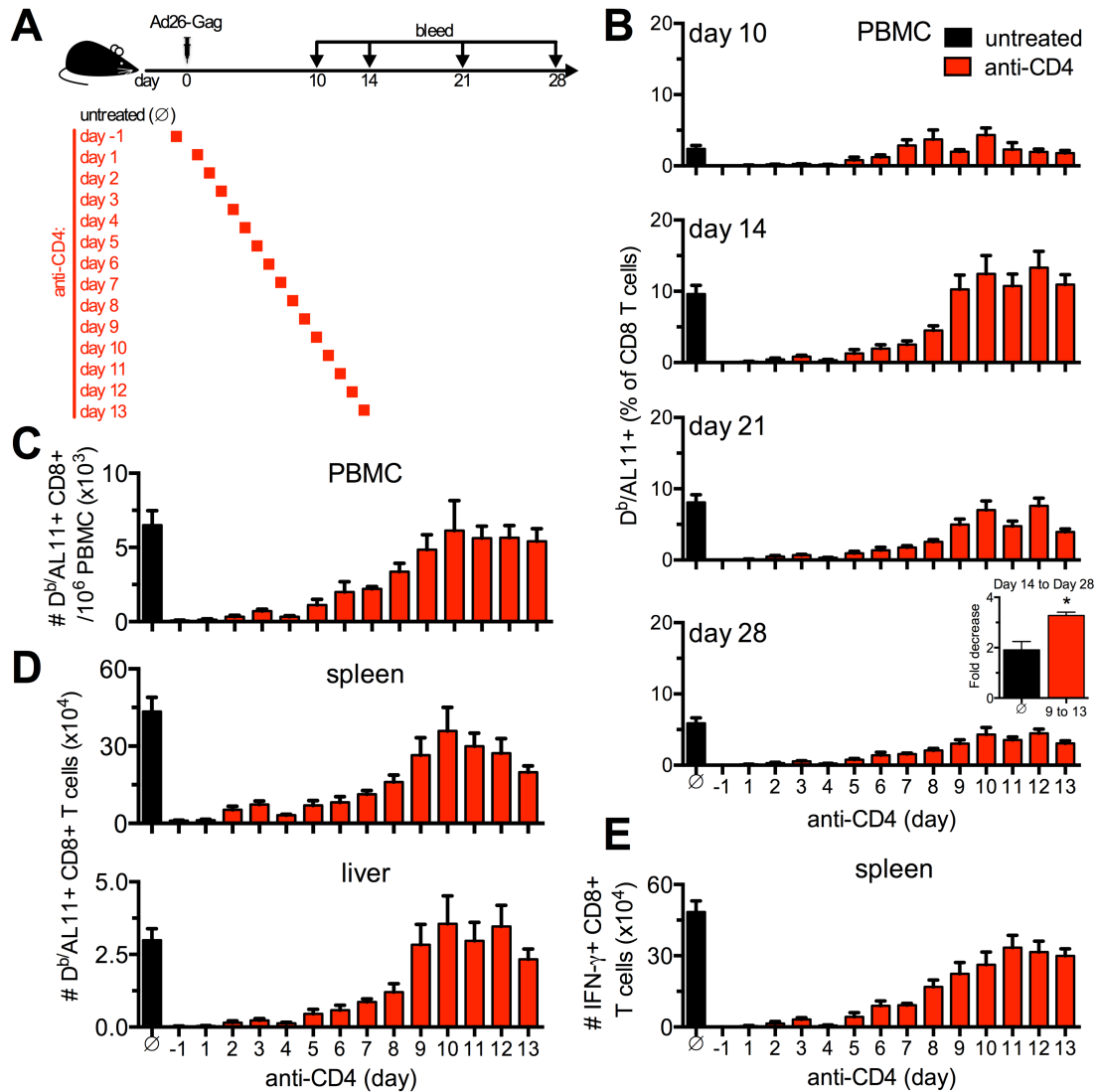


Figure 2.2. Temporal requirement for $CD4^+$ T cell help for the induction and expansion of $CD8^+$ T cell responses following Ad vaccination. (A) C57BL/6 mice were depleted of $CD4^+$ T cells by i.p. administration of mAb GK1.5 (anti-CD4) beginning on day -1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 and immunized i.m. with 10^9 vp of Ad26-Gag on day 0. Untreated controls are designated by \emptyset . (B) Mice from each treatment group were bled at the indicated time points and AL11-specific $CD8^+$ T cells were assessed by $D^b/AL11$ staining. *Inset*, the fold-decrease in the number of AL11-specific $CD8^+$ T cells in blood from day 14 to day 28 post-immunization is shown. Groups of mice administered with anti-CD4 beginning on day 9 to day 13 were pooled for analysis. (C and D) On day 28 post-immunization the total number of AL11 peptide-specific $CD8^+$ T cells per 10^6 PBMC (C) or per tissue (D) were quantified by $D^b/AL11$ staining. (E) On day 28 post-immunization the total number of AL11 peptide-specific $CD8^+$ T cells was assessed by *ex vivo* re-stimulation with AL11 peptide followed by intracellular cytokine staining for IFN- γ . Data are from $n=7-22$ /group (pooled from two to six independent experiments). Mean \pm SEM are shown; *, $P<0.001$.

Longitudinal interruption of CD4⁺ T cell help alters effector differentiation and survival of CD8⁺ T cells

We next examined the phenotype of D^b/AL11⁺ CD8⁺ T cells from mice that were depleted of CD4⁺ T cells on day 4 to day 13 post-immunization. Of note, depletion prior to day 4 resulted in insufficient antigen-specific CD8⁺ T cells for analysis. Terminal effector and memory precursor cells were differentiated by surface marker expression of KLRG1⁺ and CD127⁺, respectively, as previously reported (53-55). On day 28 post-immunization, the frequency of D^b/AL11⁺ CD8⁺ T cells that were KLRG1⁺CD127⁻ terminal effector cells was reduced in mice depleted of CD4⁺ T cells, with a corresponding increase in the frequency that were KLRG1⁻CD127⁺ memory precursor cells ($p < 0.05$; Fig. 2.3A and B). The earlier CD4⁺ T cells were depleted, the lower the frequency of terminal effector cells and the greater the frequency of potential memory precursor cells. This trend was also observed in D^b/AL11⁺ CD8⁺ T cells from the blood and liver (Fig. 2.3C and D). No differences in the proportion of D^b/AL11⁺ CD8⁺ T cells that were KLRG1⁺CD127⁺ or KLRG1⁻CD127⁻ was observed between the groups (Fig. 2.3A). Depletion of CD4⁺ T cells after day 8 did not appear to impact these phenotypic markers. Overall, there was a decrease in the absolute number of both the KLRG1⁺CD127⁻ and KLRG1⁻CD127⁺ populations of D^b/AL11⁺ CD8⁺ T cells in the spleens of mice depleted of CD4⁺ T cells prior to day 8 (Fig. 2.3E). Thus, the altered frequency of KLRG1⁺CD127⁻ and KLRG1⁻CD127⁺ cells is not due to a specific survival defect of one subset, but instead may reflect altered differentiation of CD8⁺ T cells in mice depleted of CD4⁺ T cells post-immunization.

Given the increase in the percentage of D^b/AL11⁺ CD8⁺ T cells that were KLRG1⁻CD127⁺ following CD4⁺ T cell depletion from day 4-7 post-immunization, we sought to

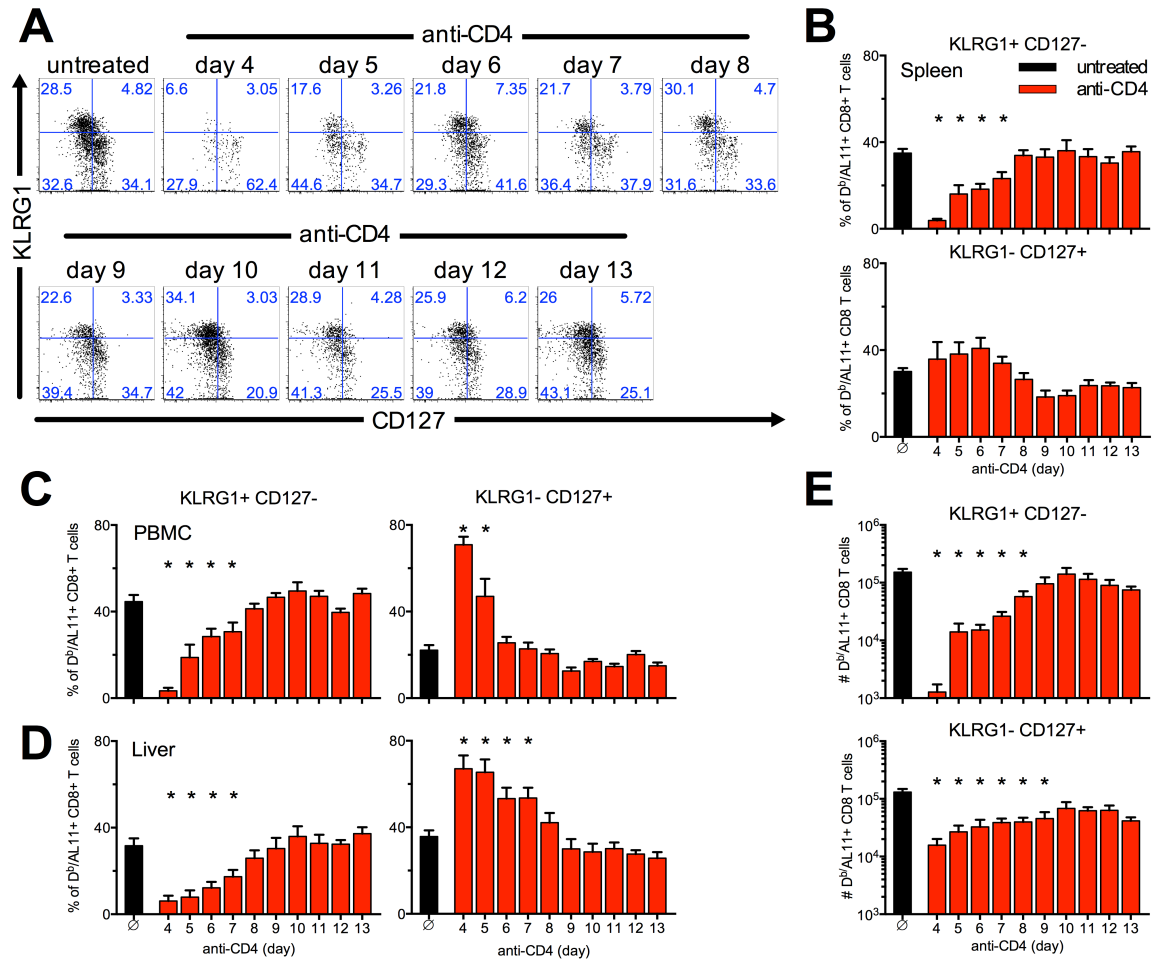


Figure 2.3. Effector differentiation of CD8⁺ T cells is dependent on CD4⁺ T cell help following Ad vaccination. C57BL/6 mice were depleted of CD4⁺ T cells by i.p. administration of mAb GK1.5 (anti-CD4) beginning on day 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or were left untreated (○), and were immunized i.m. with 10⁹ vp of Ad26-Gag on day 0. On day 28 post-immunization, animals were sacrificed and tissues were harvested. KLRG1 and CD127 expression on D^b/AL11⁺ CD8⁺ T cells was assessed. (**A and B**) Representative flow plots (**A**) and group averages (**B**) of KLRG1 and CD127 expression on D^b/AL11⁺ CD8⁺ T cells in the spleen. (**C and D**) Group summary of KLRG1 and CD127 expression of D^b/AL11⁺ CD8⁺ T cells in blood (**C**) and liver (**D**). (**E**) Absolute number of KLRG1⁺CD127⁻ or KLRG1⁻CD127⁺ D^b/AL11⁺ CD8⁺ T cells in the spleen. Anti-CD4 treatment on day -1, 1, 2, and 3 post-immunization had insufficient numbers of D^b/AL11⁺ cells for analysis. Data are from n=8-16/group (pooled from two to four independent experiments), except for PBMC anti-CD4 day 9, 10, and 12 groups which are n=3-4/group (one experiment). Mean ± SEM are shown; *, P<0.05.

determine whether CD4⁺ T cell depletion also altered the expression of other memory-associated surface markers. Expression of CD62L and CD27 has been correlated with a long-term memory phenotype (39, 56). We examined the expression of these markers on D^b/AL11⁺ CD8⁺ T cells from the spleen at day 28 post-immunization of untreated controls or animals that were depleted of CD4⁺ T cells on day 5 (reduced peak CD8⁺ T cell response and reduced terminal effector phenotype), day 8 (reduced peak CD8⁺ T cell response but normal terminal effector phenotype), or day 11 (normal peak CD8⁺ T cell response and normal terminal effector phenotype) post-immunization. Depletion of CD4⁺ T cells on day 8 or 11 post-immunization progressively led to a significant reduction in CD27 expression on D^b/AL11-specific CD8⁺ T cells ($p < 0.05$; Fig. 2.4A). Conversely, depletion of CD4⁺ T cells resulted in an increase in the fraction of D^b/AL11⁺ CD8⁺ T cells that were CD62L⁺ as compared to the untreated controls ($p < 0.03$; Fig. 2.4A), and earlier CD4⁺ T cell depletion resulted in higher CD62L expression. These data suggest that absence of CD4⁺ T cell help led to dysfunctional expression of memory-associated markers and not in fact accelerated conversion to memory precursor cells.

To characterize further the impact of CD4⁺ T cell depletion on effector phenotypes, we investigated the expression of transcriptional regulators that drive CD8⁺ T cell effector differentiation. The transcription factors T-bet, Eomesodermin (Eomes), and T cell factor 1, as well as the transcriptional regulators Blimp-1, Bcl-6, Id2, and Id3 have reported roles in dictating effector versus memory differentiation of CD8⁺ T cells (49, 50, 57-63). On day 28 post-immunization, we assessed the expression of these transcriptional regulators in sorted D^b/AL11⁺ CD8⁺ T cells from the spleen of untreated controls, or in mice depleted of CD4⁺ T cells on day 5 or day 8 post-immunization. We observed by qRT-PCR that *Tbx21* (encodes T-bet) expression by D^b/AL11⁺ CD8⁺ T cells from mice depleted of CD4⁺ T cells on day 5 was 4-fold down-

regulated relative to the untreated controls ($p=0.067$; Fig. 2.4C). This group also exhibited a 10-fold down-regulation of *Eomes* and *Bcl6* as compared to untreated controls ($p<0.04$; Fig. 2.4C). We observed only modest differences in the expression of *Prdm1* (encodes Blimp-1), *Id2*, *Id3*, and *Tcf7* (encodes T cell factor 1) (Fig. 2.4C). No significant differences were observed in mice depleted of CD4⁺ T cells on day 8 post-immunization. We confirmed the reduced expression of T-bet on D^b/AL11⁺ CD8⁺ T cells by flow cytometry from animals depleted of CD4⁺ T cells as compared to untreated controls ($p<0.001$; Fig. 2.4B). *Eomes* and T-bet have been demonstrated to act in concert to promote effector differentiation and expression of CD122 (49). Consistent with this, in animals depleted of CD4⁺ T cells on day 5 post-immunization there was decreased expression of CD122 as compared to untreated controls ($p=0.038$; Fig. 2.4A). These data suggest that the defect in effector phenotype seen following depletion of CD4⁺ T cells is consistent with aberrant expression of transcriptional regulators involved in effector CD8⁺ T cell differentiation.

Our earlier findings identified that the contraction of D^b/AL11⁺ CD8⁺ T cell responses were increased following CD4⁺ T cell depletion post-immunization (Fig. 2.2B). To investigate the mechanism of this increased contraction, we evaluated expression of the apoptotic marker Annexin V on CD8⁺ T cells on day 28 post-immunization from mice depleted of CD4⁺ T cells on day 5, 8, or 11 post-immunization. We observed that the antigen-specific CD8⁺ T cells from these mice depleted of CD4⁺ T cells exhibited increased Annexin V expression (54%, 41%, and 21%, respectively), as compared to 15% of D^b/AL11⁺ CD8⁺ T cells from the untreated control animals ($p<0.04$; Fig. 2.4D). These data suggest that CD4⁺ T cells play a key role in not only the induction but also the survival of antigen-specific CD8⁺ T cell responses (27).

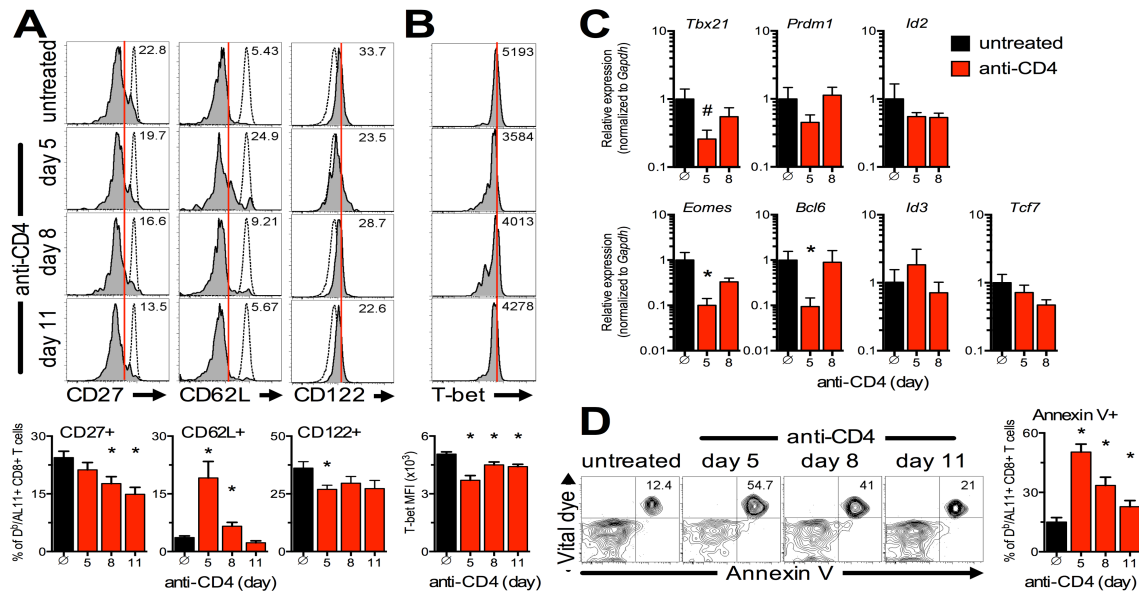


Figure 2.4. CD8⁺ T cells in mice depleted of CD4⁺ T cells exhibit an abnormal phenotype following intramuscular Ad vaccination. C57BL/6 mice were depleted of CD4⁺ T cells by i.p. administration of mAb GK1.5 (anti-CD4) on day 5, 8, or 11, or left untreated (○), and immunized i.m. with 10⁹ vp of Ad26-Gag. On day 28 post-immunization, animals were sacrificed and splenocytes were isolated. **(A and B)** Expression level of the indicated protein as percentage **(A)** or MFI **(B)** on gated D^b/AL11⁺ cells. Dashed lines indicate CD44^{lo} CD8⁺ (naïve) T cells and solid shaded histograms indicate D^b/AL11⁺ CD8⁺ T cells. Data are from n=8-16/group (pooled from two to four independent experiments). **(C)** Gene expression of sorted D^b/AL11⁺ CD8⁺ T cells from untreated animals or animals treated with GK1.5 (anti-CD4) on day 5 or day 8 post-immunization was determined by quantitative real-time PCR. Gene expression was normalized to *Gapdh*. Data are from n=4-6/group (pooled from two independent experiments). **(D)** Annexin V expression and uptake of vital dye by D^b/AL11⁺ cells was assessed. Data are from n=8/group (pooled from two independent experiments). Mean ± SEM are shown; #, P=0.067; *, P<0.05.

CD8⁺ T cells primed with reduced CD4⁺ T cell help fail to protect against lethal recombinant *Listeria monocytogenes* challenge

We next sought to determine the impact of longitudinal depletion of CD4⁺ T cells on the ability of vaccine-elicited CD8⁺ T cells to protect against a lethal recombinant *Listeria monocytogenes* challenge. To accomplish this we utilized Ad26-GP followed by challenge on day 30 following immunization with Lm-GP33 (Fig. 2.5A). Longitudinal depletion of CD4⁺ T cells following immunization with 10⁹ vp of Ad26-GP resulted in a reduced frequency of D^b/GP33⁺ CD8⁺ T cells (Fig. 2.5B), consistent with our previous findings (Fig. 2.2). No D^b/GP33⁺ CD8⁺ T cells were detected when depletion of CD4⁺ T cells was performed on day -1 or 5, with progressively less impairment following depletion of CD4⁺ T cells on day 8 or 11. Depletion of CD4⁺ T cells on day 8 post-immunization resulted in altered expression on KLRG1 and CD127 on D^b/GP33⁺ CD8⁺ T cells, and to a lesser degree when depletion of CD4⁺ T cells was performed on day 11 post-immunization (Fig. 2.5C).

Challenge of unvaccinated mice with Lm-GP33 resulted in 100% mortality by day 4 post-immunization (Fig. 2.5D). In contrast, mice immunized 30 days prior with Ad26-GP exhibited a significant delay and reduction in mortality compared to unvaccinated controls ($p < 0.0001$; Fig. 2.5D). Mice immunized with Ad26-GP and depleted of CD4⁺ T cells on day 8 (impaired frequency and altered phenotype) exhibited comparable mortality to unvaccinated control animals ($p = 0.13$) and significantly impaired protective efficacy compared to vaccinated untreated mice ($p = 0.02$; Fig. 2.5D). These data demonstrate that depletion of CD4⁺ T cells 8 days post-immunization resulted in CD8⁺ T cells that failed to protect against a lethal recombinant *Listeria* challenge.

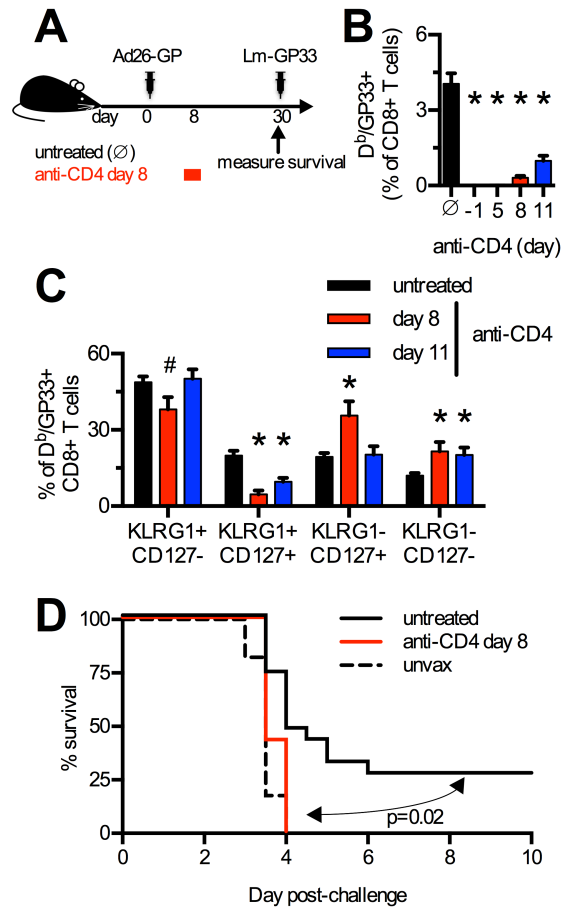


Figure 2.5. CD8 $^+$ T cells primed with reduced CD4 $^+$ T cell help fail to protect against lethal recombinant *Listeria monocytogenes* challenge. (A) C57BL/6 mice were depleted of CD4 $^+$ T cells by i.p. administration of mAb GK1.5 (anti-CD4) on day 8 or left untreated (○), and immunized with 10^9 vp of Ad26-GP. On day 30 post-immunization mice were challenged with 1.75×10^5 - 2.5×10^5 cfu of Lm-GP33. (B) Frequency of $D^b/GP33^+$ CD8 $^+$ T cells in the blood on day 30 post-immunization. (C) KLRG1 and CD127 expression on $D^b/GP33^+$ CD8 $^+$ T cells in the blood on day 30 post-immunization. (D) Survival of mice following Lm-GP33 challenge. Data are from n=16-32/group (A-C) or n=7-19/group (D) pooled from 2-4 independent experiments. Mean \pm SEM are shown; #, P=0.08; *, P<0.05.

Prolonged requirement for CD4⁺ T cell help for controlling the contraction of CD8⁺ T cell responses

Since depletion of CD4⁺ T cells on day 9-13 during the expansion phase of the immune response led to an increased contraction of antigen-specific CD8⁺ T cells (Fig. 2.2B) and increased apoptosis (Fig. 2.4D), we sought to determine how long CD4⁺ T cell help was required following immunization to regulate the contraction of antigen-specific CD8⁺ T cells. Mice were depleted of CD4⁺ T cells on day -1, 7, 14, 21, or 28 relative to immunization with 10⁹ vp of Ad26-Gag on day 0 (Fig. 2.6A). Consistent with previous experiments, depletion of CD4⁺ T cells on day -1 resulted in complete abrogation of D^b/AL11⁺ CD8⁺ T cell responses, and depletion on day 7 resulted in markedly lower D^b/AL11⁺ CD8⁺ T cell responses relative to untreated controls (Fig. 2.6B). Depletion of CD4⁺ T cells on days 14, 21, or 28 resulted in more rapid decay of the D^b/AL11⁺ CD8⁺ T cell response as compared with untreated mice as a function of the day of depletion (Fig. 2.6B). On day 90, the frequency of D^b/AL11⁺ CD8⁺ T cells in untreated control animals was 3.74% of CD8⁺ T cells while the frequency of D^b/AL11⁺ CD8⁺ T cells was significantly lower in animals depleted of CD4⁺ T cells on day -1 (0.04%, below the limit of detection; p=0.002), day 7 (1.97%; p=0.005), day 14 (2.0%; p=0.016), or day 21 (1.84%; p=0.0007), and a trend was observed in animals depleted of CD4⁺ T cells on day 28 (2.79%; p=0.11; Fig. 2.6B). When the magnitude of the response on day 90 was normalized to the peak of the response on day 14, the degree of contraction of the D^b/AL11⁺ CD8⁺ T cell responses was significantly greater in mice depleted of CD4⁺ T cells on day 14 or 21 post-immunization (p<0.05), and a trend was observed in mice depleted of CD4⁺ T cells on day 28 post-immunization (p=0.08; data not shown). Thus, depletion of CD4⁺ T cells on day 7, 14, or 21 led to an ~2-fold reduction in frequency of D^b/AL11⁺ CD8⁺ T cells on day 90 as compared to

untreated controls. However, the impact of increased contraction of vaccine-elicited CD8⁺ T cells on protective efficacy remains to be determined.

Given our previous observations of abnormal phenotypes of D^b/AL11⁺ CD8⁺ T cells following early interruption of CD4⁺ T cell help (Fig. 2.3 and 2.4), we assessed the long-term expression of KLRG1 and CD127 in animals depleted of CD4⁺ T cells on days -1, 7, 14, 21, or 28. Depletion of CD4⁺ T cells on day 7 resulted in CD8⁺ T cells that did not undergo the normal phenotypic evolution observed in the untreated controls. The frequency of D^b/AL11⁺ CD8⁺ T cells that expressed KLRG1 and CD127 failed to increase over time in the mice depleted of CD4⁺ T cells on day 7 and was significantly lower at day 50 and 90 post-immunization as compared to the untreated controls ($P < 0.05$; Fig. 2.6C). Animals depleted of CD4⁺ T cells on day 14, 21, or 28 post-immunization exhibited a less abnormal phenotypic evolution (Fig. 2.6C), consistent with the early need for CD4⁺ T cell help to program this process. These data demonstrate a prolonged role for CD4⁺ T cells in controlling the contraction of antigen-specific CD8⁺ T cell responses.

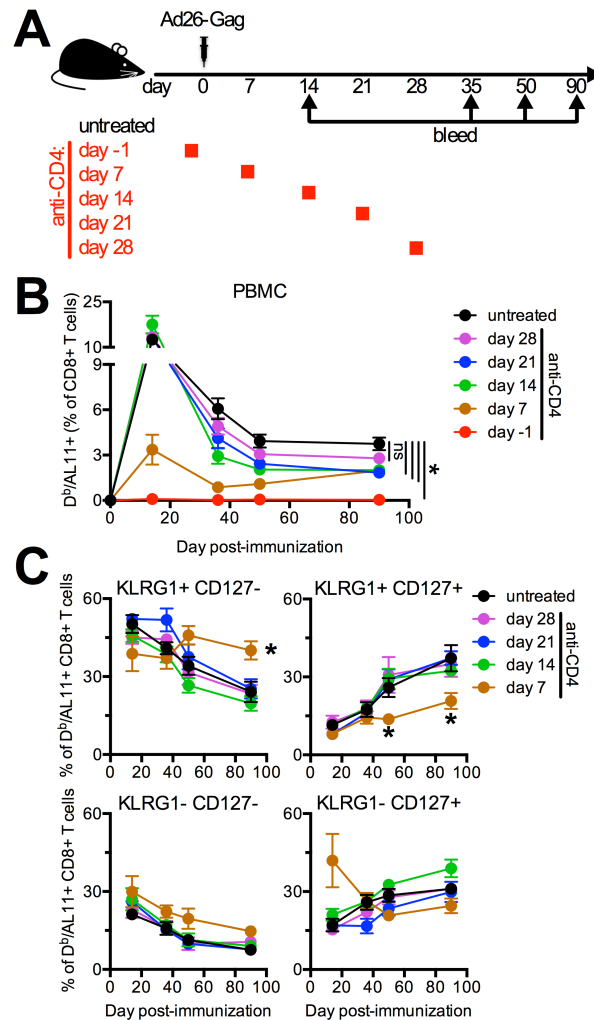


Figure 2.6. Normal contraction of the CD8⁺ T cell response requires prolonged CD4⁺ T cell help. (A) C57BL/6 mice were depleted of CD4⁺ T cells by i.p. administration of mAb GK1.5 (anti-CD4) on day -1, 7, 14, 21, or 28, or left untreated (○), and immunized i.m. with 10⁹ vp of Ad26-Gag. (B) Animals were bled longitudinally and Gag-specific CD8⁺ T cells were quantified by D^b/AL11 tetramer staining. (C) Frequency of KLRG1 and CD127 expression on D^b/AL11⁺ CD8⁺ T cells in blood. Data are from n=8-12/group (pooled from two to three independent experiments). Mean ± SEM are shown; *, P<0.05.

CD8⁺ T cells that receive at least eight days of CD4⁺ T cell help exhibit normal anamnestic potential

Given that the provision of CD4⁺ T cell help for at least eight days post-immunization resulted in CD8⁺ T cells that exhibited a largely normal phenotype, we sought to determine if these cells also had acquired the normal memory characteristic of anamnestic expansion upon antigen re-exposure. Therefore, we performed adoptive transfer experiments of AL11-specific CD8⁺ T cells from mice depleted of CD4⁺ T cells into naive animals followed by secondary antigen exposure. As outlined in Figure 2.7A, CD45.1⁺ congenic mice immunized with 10⁹ vp of Ad26-Gag were either untreated or depleted of CD4⁺ T cells on day 8, 11, or 14. On day 28 post-immunization, splenic CD8⁺ T cells were purified and equal numbers of CFSE-labeled D^b/AL11⁺ CD8⁺ T cells were transferred into CD45.2⁺ congenic mice. One day after cell transfer, recipient animals were immunized with 10⁹ vp of Ad5HVR48-Gag and the expansion of D^b/AL11⁺ CD45.1⁺ CD8⁺ T cells was evaluated. Eight days following immunization, the D^b/AL11⁺ CD8⁺ CD45.1⁺ donor T cells from untreated controls and the donor cells from mice depleted of CD4⁺ T cells expanded to nearly equivalent levels (Fig. 2.7B and D). All of the D^b/AL11⁺ CD8⁺ CD45.1⁺ donor T cells had also completely diluted CFSE by day 8 post-boost (Fig. 2.7C), which is consistent with the robust expansion of the population. These data suggest two possible options: either CD4⁺ T cells are not required to program anamnestic responses in the Ad system (17) or this CD4⁺ T cell mediated anamnestic programming occurs within eight days of immunization (27, 64, 65).

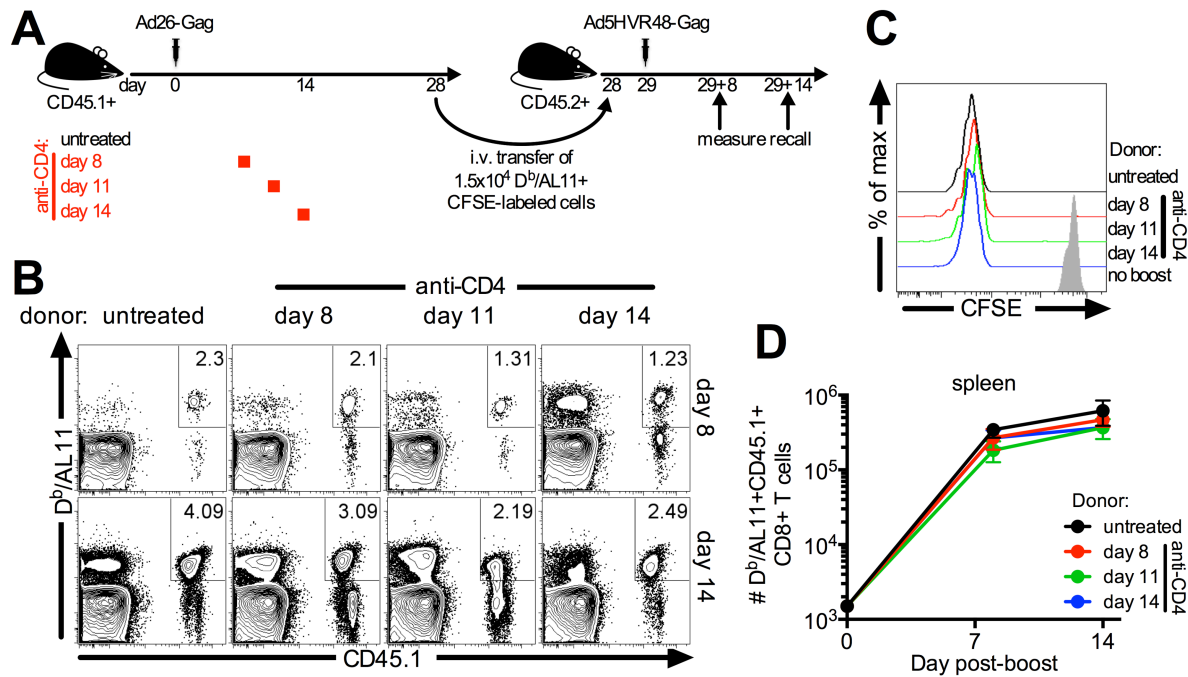


Figure 2.7. CD8⁺ T cells primed with at least eight days of CD4⁺ T cell help proliferate upon secondary antigen exposure. (A) CD45.1⁺ mice were depleted of CD4⁺ T cells by i.p. administration of mAb GK1.5 (anti-CD4) on day 8, 11, or 14, or left untreated, and immunized i.m. with 10^9 vp of Ad26-Gag (day 0). On day 28 post-immunization, CD8⁺ T splenocytes were pooled, enriched by negative selection, and labeled with CFSE. 1.5×10^4 D^b/AL11⁺ CD8⁺ T cells were transferred by tail vein injection into naïve CD45.2⁺ recipients. One day following transfer, animals were immunized i.m. with 10^9 vp of Ad5HVR48-Gag. (B) Representative plots of D^b/AL11⁺ CD45.1⁺ donor cells as a fraction of CD8⁺ T cells in the spleen on day 8 or 14. (C) Representative histograms of CFSE dilution in the donor D^b/AL11⁺ CD45.1⁺ CD8⁺ T cell population on day 8 post-boost (open histogram) or the donor CD45.1⁺ CD8⁺ T cell population with no boosting immunization (solid histogram). (D) Absolute number of D^b/AL11⁺ CD45.1⁺ CD8⁺ T cells in the spleen on day 8 or 14 post-immunization. Frequency of donor D^b/AL11⁺ CD45.1⁺ CD8⁺ T cells at the time of immunization (day 0) is based on 10% engraftment. Data are from $n=6-13$ recipient mice/group on day 8 (pooled from three independent experiments) and $n=5$ recipient mice/group on day 14 (from one experiment). Mean \pm SEM are shown.

The CD40 signaling pathway is not critical for initial CD8⁺ T cell priming but is required for anamnestic CD8⁺ T cell expansion

Several reports have identified the CD40 signaling pathway as a major mechanism for the provision of CD4⁺ T cell help, both for the generation of primary CD8⁺ T cell responses and for the programming of anamnestic responses (21, 31-33, 66, 67). Therefore, we evaluated the importance of this signaling pathway for the generation of Ad vector-elicited CD8⁺ T cell responses. C57BL/6, CD40L KO, and CD40 KO mice were immunized intramuscularly with 10⁹ vp of Ad26-Gag. On day 28 post-immunization, no significant differences in the number of D^b/AL11⁺ CD8⁺ T cells between wild type and CD40 pathway deficient (CD40L KO or CD40 KO) animals were observed (Fig. 2.8A). Consistent with this, the absolute number of Gag-specific CD8⁺ T cells that secreted IFN-γ was comparable between the wild type and CD40L KO and CD40 KO animals (Fig. 2.8B). Moreover, splenic D^b/AL11⁺ CD8⁺ T cells from wild type, CD40L KO, and CD40 KO animals expressed equivalent levels of KLRG1 and CD127 (Fig. 2.8C). Collectively, these data suggest that signaling via CD40 is not critical for the initial generation of Ad vector-elicited primary CD8⁺ T cell responses.

To determine the role of CD40 signaling in the generation of anamnestic CD8⁺ T cell responses, Ad26-Gag primed C57BL/6, CD40L KO, and CD40 KO animals were boosted with 10⁹ vp of Ad5VHR48-Gag on day 56. The frequency of D^b/AL11⁺ CD8⁺ T cells in the blood prior to priming was equivalent between groups (Fig. 2.8D). Upon boosting, the D^b/AL11⁺ CD8⁺ T cell population in wild type animals expanded 15-fold by day 7 post-boost and had begun to contract by day 14 (Fig. 2.8D and E). However, in CD40L KO and CD40 KO animals, the D^b/AL11⁺ CD8⁺ T cell population expanded significantly less by day 7 post-boost than in wild type animals (P<0.03; Fig. 2.8D and E).

Finally, to determine if signaling via CD40 was required at the time of priming or boosting, on day 50 equal numbers of D^b/AL11⁺ CD8⁺ T cells from wild type or CD40L KO Ad26-Gag-immunized animals were transferred into congenic (CD45.1⁺) naïve hosts (Fig. 2.8F). Following an Ad5HVR48-Gag immunization, the recall potential of donor D^b/AL11⁺ CD8⁺ T cells was assessed. The number of donor D^b/AL11⁺ CD8⁺ T cells from CD40L KO animals was significantly reduced following Ad5HVR48-Gag immunization in peripheral blood, spleen, and liver compared to donor D^b/AL11⁺ CD8⁺ T cells from wild type animals ($P < 0.05$; Fig. 2.8F). These data suggest that while CD40-derived signals are not required for the generation of a primary CD8⁺ T cell response, CD40 is critical for programming anamnestic potential upon secondary antigen challenge. Collectively these experiments demonstrate a prolonged and multifaceted role for CD4⁺ T cell help to generate optimal Ad vector-induced CD8⁺ T cell responses (Fig. 2.9).

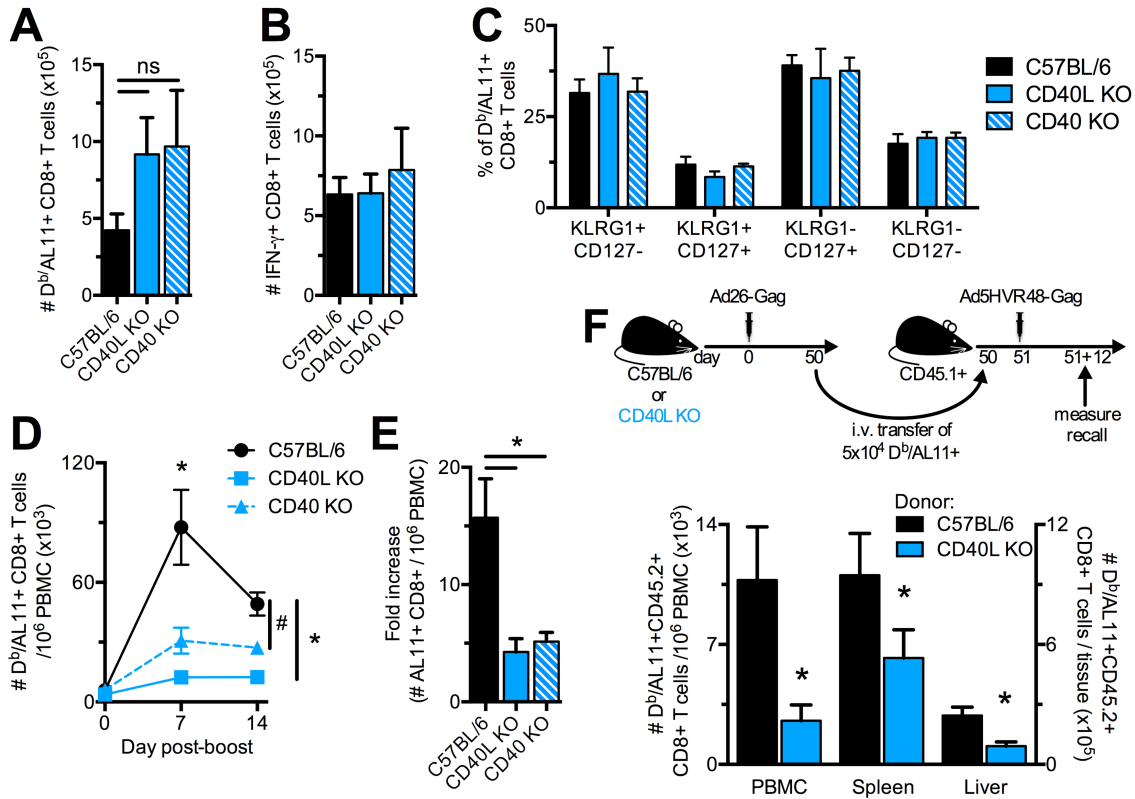


Figure 2.8. CD40 signaling is required for programming CD8⁺ T cells with anamnestic potential. (A-C) C57BL/6, CD40L KO, and CD40 KO animals were immunized i.m. with 10⁹ vp of Ad26-Gag (n=8/group pooled from two independent experiments). (A) The number of D^b/AL11⁺ CD8⁺ T cells in the spleen of immunized animals on day 28 post-immunization. (B) Splenocytes were harvested on day 28 post-immunization and the number of IFN-γ⁺ CD8⁺ T cells was quantified following stimulation with an overlapping SIV Gag peptide pool. (C) KLRG1 and CD127 expression were assessed on splenic D^b/AL11⁺ CD8⁺ T cells on day 28 post-immunization. (D and E) C57BL/6, CD40L KO, and CD40 KO animals were immunized i.m. with 10⁹ vp of Ad26-Gag and on day 56 post-prime were boosted with 10⁹ vp of Ad5HVR48-Gag (n=4/group from one experiment). (D) The frequency of D^b/AL11⁺ CD8⁺ T cells per 10⁶ PBMC following the boost was assessed. (E) The fold-change in frequency of D^b/AL11⁺ CD8⁺ T cells per 10⁶ PBMC from one day pre-boost to day 7 post-boost was assessed. (F) C57BL/6 or CD40L KO animals were immunized intramuscularly with 10⁹ vp of Ad26-Gag. On day 50 post-immunization, 5x10⁴ D^b/AL11⁺ CD8⁺ T cells were enriched by negative selection and transferred intravenously to congenically marked (CD45.1⁺) naïve recipients (n=8-9/group from two independent experiments). One day post-transfer, recipient mice were immunized i.m. with 10⁹ vp of Ad5HVR48-Gag. On day 12 post-secondary immunization, the number of donor D^b/AL11⁺ CD45.2⁺ CD8⁺ T cells in PBMC, spleen, and liver was assessed. Mean ± SEM are shown; #, P=0.06; *, P<0.05.

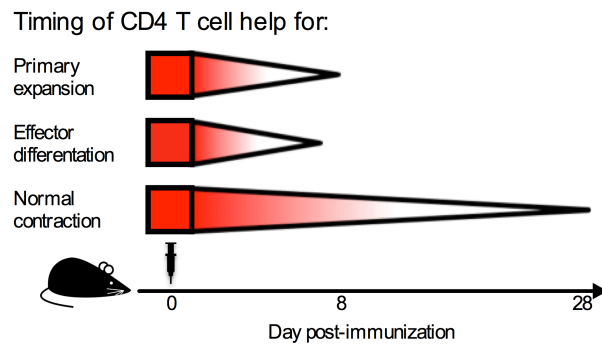


Figure 2.9. Schematic representation of longitudinal requirement for CD4⁺ T cell help. Summary of longitudinal requirement for CD4⁺ T cell help for the induction of CD8⁺ T cell responses, effector differentiation, and normal contraction following Ad vector vaccination. The requirement for CD4⁺ T cell help was prolonged but waned over time.

DISCUSSION

In this study we demonstrate that immunization with Ad vectors requires CD4⁺ T cell help for the induction and optimal sustainment of CD8⁺ T cell responses not only at the time of priming but also for a prolonged period of time following immunization. We observed that CD4⁺ T cell help was required for eight days post-immunization for primary induction of CD8⁺ T cell responses and normal effector differentiation. Moreover, CD4⁺ T cells were critical for four weeks post-immunization for controlling the contraction of CD8⁺ T cell responses. CD40 signaling was required to program CD8⁺ T cells with anamnestic potential but did not appear critical for initial CD8⁺ T cell priming. Taken together, these data demonstrate that the need for CD4⁺ T cell help to generate Ad vector-induced CD8⁺ T cell responses is multi-faceted and prolonged over time (Fig. 2.9).

Our data suggest that Ad vector-based vaccines and potentially also other vaccines may exhibit reduced cellular immunogenicity in patients with reduced CD4⁺ T cell function, such as in patients with advanced AIDS or following hematopoietic stem cell transplantation (HSCT) as a result of insufficient CD4⁺ T cell help. Consistent with this hypothesis, HIV-1-infected patients and HSCT patients have been reported to develop impaired antibody responses following vaccination against influenza virus, hepatitis B virus, *Haemophilus influenza* type B, tetanus-reduced diphtheria-reduced pertussis (Tdap), and yellow fever virus (68-72). Most previous studies have focused on vaccine-elicited antibody responses, and the requirement for CD4⁺ T cell help to generate CD8⁺ T cell responses by vaccination has remained poorly understood. Our data suggest that prolonged CD4⁺ T cell help is required for the induction and sustainment of optimal vaccine-elicited cellular immune responses.

Prior studies have reported that CD4⁺ T cell help is required at the time of priming to generate robust Ad vector-elicited CD8⁺ T cell responses (17, 18). However, the timing of CD4⁺ T cell help for the development of primary CD8⁺ T cell responses has not been previously reported to the best of our knowledge. Our data confirm and extend the observation of a requirement for CD4⁺ T cell help at the time of vaccine priming with multiple serotypes of Ad vectors. Longitudinal depletion of CD4⁺ T cells identified a prolonged role for CD4⁺ T cell help in the expansion and contraction of CD8⁺ T cell responses. In contrast, Yang and colleagues (17) did not detect any differences in CD127, CD62L, and CD122 between Ad5-elicited antigen-specific CD8⁺ T cells that did or did not receive CD4⁺ T cell help. This difference may reflect the inherent difference between the ability of Ad5- and Ad26-induced CD8⁺ T cells to express memory markers, as has been recently reported (36, 73). Furthermore, the largest differences in phenotype observed in the current study related to KLRG1 expression, which was not assessed in this prior study (17).

A number of reports have identified that pathogens/vaccines can be broadly divided into two categories based on whether CD4⁺ T cell help is required for induction of primary CD8⁺ T cell responses [reviewed in (30)]. Most candidate vaccine platforms (including adenovirus vectors) fall into the category of requiring CD4⁺ T cell help for the induction of primary CD8⁺ T cell responses. In contrast, the widely used viral infection model LCMV generates a primary CD8⁺ T cell response in the absence of CD4⁺ T cell help (23). Following LCMV infection of CD4 KO animals, the CD8⁺ T cell response is initially of normal magnitude but has impaired memory potential characterized by T-bet-mediated suppression of CD127, CD27, and CD62L expression (74). In contrast, we identified that following Ad vector immunization, the absence of CD4⁺ T cells resulted in impaired effector differentiation (KLRG1 and CD122) and atypical

memory marker expression (CD127, CD27, and CD62L), and this corresponded to a decrease in T-bet and Eomes expression. These data suggest that in the absence of CD4⁺ T cell help adenovirus vector-induced CD8⁺ T cell responses fail to receive the necessary signals to up-regulate T-bet and Eomes and hence do not properly initiate the program of effector differentiation. Thus it appears that in addition to their ability to drive CD4⁺ T cell-independent expansion, a fundamental difference between these two classes of antigenic stimuli is their intrinsic ability to induce effector T cell differentiation.

Impaired maintenance of the CD8⁺ T cell population in the absence of CD4⁺ T cells has been reported in other systems (27, 29, 65). In these prior studies, CD4⁺ T cells were removed at only a single time post-infection and thus the temporal requirements for CD4⁺ T cell help could not be assessed. Consistent with these reports, we observed accelerated contraction of CD8⁺ T cells following longitudinal depletion of CD4⁺ T cells. We extended these observations by demonstrating a prolonged requirement for CD4⁺ T cell help that lasted at least four weeks and declined over time. Future studies will be required to determine how this accelerated contraction alters protective efficacy of these vaccine elicited CD8⁺ T cells.

CD4⁺ T cells have a well-established role in programming memory CD8⁺ T cells to be capable of expansion upon secondary antigen exposure, and CD40-derived signals are a major mechanism of this process (20, 27, 31-33, 64). However, a previous report has demonstrated in the Ad system that CD8⁺ T cells have unimpaired anamnestic potential when primed in the absence of CD4⁺ T cells (17). Given no detectable AL11-specific CD8⁺ T cell responses in our system when CD4⁺ T cells were depleted prior to priming it was not possible to directly investigate a role for CD4⁺ T cell programming of anamnestic potential at priming, but we observed no defect in anamnestic expansion when CD4⁺ T cells were depleted after priming.

However, we observed impaired recall responses in CD8⁺ T cells from mice that lacked the CD40 signaling pathway. There are two possible conclusions from these data. First, anamnestic programming occurs rapidly following priming (27, 64, 65). Alternatively, anamnestic programming in the Ad system occurs independent of CD4⁺ T cell help (17), but still requires CD4 T cell-independent CD40 signaling, which has been reported following influenza infection (75). Future experiments will be required to fully elucidate the relationship between CD4⁺ T cell help, CD40 signaling, and the programming of anamnestic potential following Ad vector immunization.

This study illustrates the multi-faced role of CD4⁺ T cells in the development and maintenance of Ad vector-induced CD8⁺ T cell responses. CD4⁺ T cell help at different points in time was required for CD8⁺ T cell priming, effector differentiation, and regulating contraction (Fig. 2.9). The relative importance of CD4⁺ T cell help gradually waned over time following immunization, but was required for eight days for CD8⁺ T cell priming and 28 days for regulating contraction. Thus it appears that CD4⁺ T cells do not simply program functional CD8⁺ T cells through a single or transient signaling event, but instead modulate CD8⁺ T cell frequency and functionality via an active process over a prolonged period of time. Future efforts to optimize CD4⁺ T cell responses, especially in situations of impaired CD4⁺ T cell frequency such as HIV infection, may lead to increased immunogenicity and durability of CD8⁺ T cell responses elicited by Ad vector-based vaccines and possibly other vaccine platforms as well.

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Chapter 3: Immediate Dysfunction of Vaccine-Elicited CD8⁺ T Cells Primed in the Absence of CD4⁺ T Cells

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ABSTRACT

CD4⁺ T cell help is critical for optimal CD8⁺ T cell expansion after priming. However, a role for CD4⁺ T cells in regulating the initial steps of CD8⁺ T cell effector differentiation is not well established. Here we demonstrate that absence of CD4⁺ T cells at the time of adenovirus vector immunization leads to immediate CD8⁺ T cell dysfunction. Unhelped CD8⁺ T cells exhibit reduced *ex vivo* cytotoxicity and decreased capacity to produce cytokines. These cells express elevated levels of inhibitory receptors and exhibit transcriptomic exhaustion and anergy profiles by gene set enrichment analysis. AP-1-independent NFAT signaling appears to be important in driving this dysfunction. This state is imprinted within 3 days of immunization. Impaired expansion and effector differentiation of unhelped CD8⁺ T cell is caused by reduced IL-2 and elevated PD-1 signaling. This study identifies a novel, previously undescribed role of CD4⁺ T cells to prevent immediate dysfunction and features of exhaustion in CD8⁺ T cells following antigen priming.

INTRODUCTION

CD4⁺ T cells are key regulators of CD8⁺ T cell function, as the absence of CD4⁺ T cell help at priming leads to impaired responses, including reduced expansion (1-3), hyper-effector differentiation (4, 5), and reduced anamnestic potential (6-8). Induction of primary CD8⁺ T cells by several non-inflammatory antigens requires CD4⁺ T cell help, which is mediated by CD40 signaling (2, 9, 10). Replication-incompetent Adenovirus (Ad) vector vaccines are important clinically relevant tools for probing immune regulatory pathways, and require CD4⁺ T cell help for the induction of robust primary CD8⁺ T cell responses (11-14). However, this help is provided through a CD40-independent mechanism (11). Thus, further work is required to identify the mechanism of CD4⁺ T cell help following immunization with viral vector vaccines.

Following vaccination or infection that is acutely controlled, CD8⁺ T cells typically differentiate into two distinct highly functional effector and memory populations (15). A role for CD4⁺ T cells in regulating CD8⁺ T cell differentiation is not well defined. In the lymphocytic choriomeningitis model unhelped CD8⁺ T cells differentiate into a hyper-effector state due to excessive T-bet expression (4), and in the vaccinia virus model conflicting reports identify no role for CD4⁺ T cells in regulating effector CD8⁺ T cell differentiation (16) or hyper-effector differentiation of unhelped CD8⁺ T cells (5). In contrast, following Ad vector immunization, unhelped CD8⁺ T cells fail to express effector phenotype markers (11). Whether these phenotypic defects reflect altered functionality remains to be addressed. Thus, further work is required to fully understand what role CD4⁺ T cells have in regulating CD8⁺ T cell effector function upon viral vector immunization, and how CD4⁺ T cells regulate this process.

In this study we sought to clearly define the role of CD4⁺ T cells in regulating CD8⁺ T cell effector differentiation, and how this is accomplished. We demonstrate that in the absence of

CD4⁺ T cell help CD8⁺ T cells induced by vaccination with a replication-incompetent viral vector differentiate to a dysfunctional state, which has many of the phenotypic and functional characteristics of CD8⁺ T cell exhaustion. However, in contrast to the progressive process of chronic infection-induced exhaustion, this dysfunction is programmed at priming and defects in differentiation are observed within days of immunization. CD8⁺ T cells express both exhaustion and anergy transcriptional signatures, and this includes exhaustion and anergy signatures for AP-1-independent NFAT target genes. These data are consistent with a model in which excessive AP-1-independent NFAT signaling is critical for driving dysfunctional differentiation. Functionally, experimental modulation of either PD-1 or IL-2 signaling during priming partially prevents dysfunctional differentiation and rescues effector functionality. In sum, we identify a novel immediate role for CD4⁺ T cells in programming effector differentiation and preventing immediate exhaustion-like dysfunction of CD8⁺ T cells following viral vector immunization.

MATERIALS AND METHODS

Mice and vectors. Six- to ten-week-old C57BL/6, B6.SJL-ptprc^a (CD45.1⁺), B6.129S2-Cd4^{tm1Mak}/J (CD4 KO), B6.129S2-H2^{dIAb1-Ea}/J (MHC II KO), C57BL/6-Tg(TcrαTcrβ)1100Mjb/J (OT-I), and B6.Cg-Tg(TcrαTcrβ)425Cbn/J (OT-II) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Thymectomized animals underwent adult thymectomy at the Jackson Laboratory (Bar Harbor, ME). CD45.1/2⁺ OT-I TCR-Tg and B6.129S4-Il2ra^{tm1Dw}/J (CD25 KO) OT-I mice were bred in house. E1/E3 deleted Ad5-SIINFEKL-Luc, Ad5-tTA-SIINFEKL-Luc, Ad5-OVA, and Ad5HVR48(1-7)-Gag have been previously described (34, 58-60). Mice were immunized intramuscularly with 10⁹ viral particles of the indicated vector in the quadriceps using 100 μl divided equally between the two legs. Ad5-tTA-SIINFEKL-Luc was used at 10¹⁰ viral particles per mouse. NYVAC-Env has been previously described (61) and was used at 10⁶ pfu per mouse. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Beth Israel Deaconess Medical Center.

Monoclonal antibody administration. The monoclonal αCD4 antibody (GK1.5; BioXcell) was administered by two IP injections of 500 μg one day prior to immunization and on the day of immunization. To maintain CD4 T cell depletion, this injection regimen was repeated every 21 days following immunization. αPD-L1 (10F.9G2; BioXCell), αPD-1 (29F.1A12; BioLegend), or isotype IgG2b (clone LTF-2; BioXCell) were administered by 200 μg IP injections every third day, as previously described (35).

Recombinant IL-2 administration. Mouse recombinant IL-2 (R&D Systems) resuspended in 0.1% normal mouse serum was given 1 μg IP twice daily from day 3 to 12, per published dosing protocols (41, 62).

Doxycycline administration. 500 µg of doxycycline (MP Biomedicals) was injected IP on day 14 as previously described (34). Mice were subsequently given food containing 625 mg/kg doxycycline (Harlan Teklad) to maintain doxycycline levels long-term.

Tissue processing. Single cell suspensions of splenocytes and lymph nodes were generated by grinding the tissue through a 70 µm filter (Fisher Scientific). Tissue red blood cells were lysed by Ammonium-Chloride-Potassium (ACK) treatment for three minutes. Cellular debris was further clarified by passage through a 30 µm filter (Miltenyi Biotec). For purification of peripheral blood mononuclear cells (PBMC) Ficoll-Histopaque density centrifugation was performed at 1900 rpm for 20 min.

Flow cytometry. Identification of antigen-specific cells by MHC class I tetramer staining was performed using either H-2D^b tetramer loaded with the immunodominant AL11 peptide (AAVKNWMTQTL) of SIV_{mac239} Gag (63) or H-2K^b tetramer loaded with the immunodominant OVA₂₅₇₋₂₆₄ (SIINFEKL) epitope from chicken ovalbumin (64). Biotinylated class I monomers were kindly provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Background staining of cells from naïve animals was ≤0.1%. Surface staining was performed for 30 minutes at 4 °C. The following antibodies were used for staining: αCD8α (53-6.7), -CD4 (RM4-5), -CD44 (IM7), -CD45.2 (104), -CD45.1 (A20), -TCRα V2 (B20.1), -CD127 (A7R34), -KLRG1 (2F1), -CD27 (LG.3A10), -CD43 (1B11), -PD-1 (RMP1-30), -Tim-3 (RMT3-23), -LAG-3 (C9B7W), -2B4 (eBio244F4), -CD25 (PC61), -CD28 (37.51), -OX-40 (OX-86), -4-1BB (17B5), -CD71 (RI7217), -CD98 (RL388), -CD11a (2D7), and -CD69 (H1.2F3). For detection of Granzyme B, cells were surface stained and then permeabilized for 15 min at room temperature with Cytofix/Cytoperm (BD Biosciences). Permeabilized cells were subsequently stained with αGranzyme B (GB11). Ki-67 detection was performed using BD Perm Buffer 2 (BD

Biosciences) for 15 min at room temperature followed by α Ki-67 (B56) staining. For intracellular cytokine staining, cells were incubated for 5 hrs at 37 °C with 2 μ g/ml of OVA₂₅₇₋₂₆₄ peptide (AnaSpec) or 1 μ g/ml of overlapping SIV_{mac239} Env peptide pool (NIH AIDS Reagent Program). At the time of peptide incubation, Brefeldin A and Monensin (BD Biosciences) and α CD107a (ID4B) were added. After peptide incubation cells were surface stained, washed, permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4 °C, and subsequently stained with α IFN- γ (XMG1.2) and -TNF- α (MP6-XT22). All antibodies were purchased from BD Biosciences, eBioscience, BioLegend, or Life Technologies. Dead cells were excluded by use of vital exclusion dye (Life Technologies). Annexin V staining was performed using an Annexin V staining kit (BioLegend). Samples were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v9.7.2 (Treestar).

***ex vivo* killing assay.** *ex vivo* cytotoxic potential of CD8⁺ T cells was measured as previously described (65). Briefly, EL-4 cells (ATCC) were pulsed with 2 μ M OVA₂₅₇₋₂₆₄ peptide (AnaSpec) or no peptide for 1hr at 37°C. Peptide pulsed cells were stained with CFSE (Life Technologies) and unpulsed cells were stained with CellTrace Violet (Life Technologies) per manufacturers instructions. CD8⁺ T cells were enriched by negative selection using the EasySep Mouse CD8⁺ T cell enrichment kit (StemCell Technologies). The number of effector cells were determined by quantification of K^b/OVA⁺ CD8⁺ T cells and the appropriate numbers were added and the mixtures were incubated for 5 hrs at 37 °C. Following incubation, cells were stained with a vital exclusion dye (Life Technologies) to exclude dead cells. Fixed cells were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v9.7.2 (Treestar). Specific lysis was calculated as $100 - [100 \times (\% \text{ survival} / \text{average } \% \text{ survival in absence of effector cells})]$.

***in vivo* luciferase imaging.** Quantification of luciferase transgene expression was performed as previously described (60, 66). Briefly, hair was removed from the hind legs and ventral posterior area of the mouse. Mice were injected IP with 150 μ l of 30 mg/ml Luciferin (Caliper Life Sciences) per manufacturers instructions. Mice were anesthetized by 4% isoflurane inhalation and maintained under anesthesia by nose cone and 1.5% isoflurane. Luminescence was quantitated using an IVIS Lumina II charge-coupled device imaging system and Living Image software (Caliper Life Sciences). Image integration time was 240 sec, f/stop was 1.2 and binning was “large”.

Microarray. Gene expression profiling was performed as previously described (67, 68). Briefly, CD8⁺ T cells were enriched by negative selection using the CD8⁺ T cell isolation kit II (Miltenyi Biotec). D^b/AL11⁺ CD44⁺ CD8⁺ T cells were subsequently sorted to >95% purity on a FACS Aria (BD Biosciences). Sorted cells were stored at -80 °C in 1ml of TRIzol (Life Sciences). RNA extraction was performed using the RNAdvance Tissue Isolation kit (Agencourt) per manufacturers instructions. The cDNA synthesis was performed using the Ovation Pico WTA v2 kit (NuGEN) following manufacturers instructions. Proper amplification of cDNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and performed by the Harvard Biopolymers Facility. To confirm optimal amplification of mRNA and absence of contaminating gDNA (data not shown), qRT-PCR for *Rn18s* (RefSeq: NR_003278.2) and mouse RT² qPCR primer control (QIAGEN) was performed using the SYBR Green Quantification System (QIAGEN). Data were acquired on a StepOnePlus Real-Time PCR System (Applied Biosystems). Cycle conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. cDNA was subsequently fragmented and biotinylated using an Encore Biotin Module 4200 (NuGEN). cDNA was hybridized to Mouse Genome 430 v2.0 chip

(Affymetrix) at the Microarray Core of Dana Farber Cancer Institute. RMA method was used to process data image files using GenePattern (Broad Institute).

Functional enrichment and network analysis. Differential gene expression was determined using GENE-E v3.0.163 (Broad Institute). Functional enrichment, pathway, and network analyses were performed using genes ranked by adjusted *P* values, fold change, and modified *t*-statistics from limma analysis (69). Gene set enrichment analysis (GSEA) was performed using GSEA software (<http://www.broadinstitute.org/gsea>). All 45,282 probes, ranked by limma-derived modified *t*-statistics, were imported into the GSEA software. We tested for the enrichment of C2 and C7 and C3.tft Molecular Signatures Database. GSEA was run according to default parameters: probes for the same gene were collapsed into a single gene symbol (identified by its HUGO gene symbol), permutation number = 1000, and permutation type = “gene sets.” By convention, a nominal p-value of <0.05 was used as the cutoff value for significance. Enriched gene sets were grouped into modules according to shared leading edge genes using the cytoscape plugin EnrichmentMap (<http://www.cytoscape.org/>) by calculating the Jaccard index between pathways using a threshold of 0.50. The Jaccard index characterizes the overlap between two gene sets, relative to the size of their union and stratifies gene sets that correspond to similar biological processes.

Statistical analysis. Statistical analysis was performed using a two-tailed non-parametric Mann-Whitney U test in Prism v6.0f (GraphPad Software Inc.).

Accession Numbers. Microarray data is accession number GEO: GSE73001 in the NCBI GEO database.

RESULTS

Depletion of CD4⁺ T cells at the time of immunization results in aberrant CD8⁺ T cell differentiation with decreased effector function.

We utilized an Ad5-based immunization regimen to investigate the impact of CD4⁺ T cell help on CD8⁺ T cell responses, because this vaccination regimen generates small, but measurable, CD8⁺ T cell responses in the absence of CD4⁺ T cell help (11-13). Mice were depleted of CD4⁺ T cells by two sequential doses of the α CD4 antibody (clone GK1.5) beginning on the day prior to immunization and CD4⁺ T cell depletion was maintained by re-administration of the antibody every 21 days. This regimen led to complete and sustained depletion of CD4⁺ T cells (Fig. 3.S1A – see Appendix for Supplemental Figures). Following intramuscular immunization with Ad5-SIINFEKL-Luc, there was a 10-fold reduction in the frequency of K^b/OVA⁺ CD8⁺ T cells in the blood of α CD4 treated mice at the peak of the response compared to untreated control animals (Fig. 3.1A). A delay in peak expansion of the antigen-specific CD8⁺ T cell responses from day 10 to day 14, and an increased contraction of the responses in the absence of CD4⁺ T cells was also observed. A similar reduction in the frequency of K^b/OVA⁺ CD8⁺ T cells was observed in the spleen of α CD4 treated mice compared to untreated controls (Fig. 3.S1B).

We first sought to determine if the absence of CD4⁺ T cell help at the time of priming altered the effector CD8⁺ T cell differentiation. Differentiation into memory precursor or terminal effector cells was determined by the expression of CD127 and KLRG1, respectively (17-19). On day 14 post-immunization, K^b/OVA⁺ CD8⁺ T cells from α CD4 treated mice exhibited a significant impairment in KLRG1 upregulation, and approximately 50% of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells were undifferentiated KLRG1^{lo}CD127^{lo} cells (Fig. 3.1B), and this impaired differentiation was maintained long-term ($p < 0.05$; Fig. 3.S1C). A recent report has

identified an alternative scheme for identifying effector phenotype cells as $CD43^{lo}CD27^{lo}$ (20). This $CD43^{lo}CD27^{lo}$ subset was also significantly reduced in $\alpha CD4$ treated mice compared to controls ($P < 0.01$; Fig. 3.S1D). Consistent with these phenotypic data, we observed decreased expression of Granzyme B ($P < 0.01$; Fig. 3.1C) and decreased CD107a expression (upon cognate peptide restimulation; $P < 0.01$; Fig. 3.1D) on K^b/OVA^+ $CD8^+$ T cells from $\alpha CD4$ treated mice. Furthermore, in a 5 hr *ex vivo* cytotoxicity assay, K^b/OVA^+ $CD8^+$ T cells from $\alpha CD4$ treated mice displayed a major defect in cytotoxicity (Fig. 3.1E). These cytotoxic defects resulted in a failure to clear transgene expressing cells in $\alpha CD4$ treated mice ($P < 0.01$; Fig. 3.S1E), consistent with a previous report (12). Collectively, these data demonstrate a major defect in $CD8^+$ T cell differentiation in the absence $CD4^+$ T cell help.

Given the impaired effector phenotype and cytotoxicity of $CD8^+$ T cells primed without $CD4^+$ T cell help, we sought to determine if these cells displayed alterations in their ability to secrete cytokines. There was a significant reduction in the frequency and absolute number of $CD8^+$ T cells that produced IFN- γ upon *ex vivo* restimulation in mice treated with $\alpha CD4$ antibody compared to untreated control mice on day 14 (12-fold reduction), day 45 (15-fold reduction), and day 80 (15.5-fold reduction) post-immunization ($P < 0.001$; Fig. 3.1F and G). The frequency of antigen-specific $CD8^+$ T cells that express IFN- γ was substantially reduced in $\alpha CD4$ treated mice ($P < 0.001$; Fig. 3.1H), and these cells produced less per-cell IFN- γ than did cells from untreated control animals ($P < 0.05$; Fig. 3.1I). Finally, the fraction of IFN- γ^+ $CD8^+$ T cells from $\alpha CD4$ treated mice that were capable of co-producing TNF- α was significantly reduced compared to IFN- γ^+ $CD8^+$ T cells from untreated control animals ($P < 0.001$; Fig. 3.1J). These results were confirmed using CD4 knockout (KO) and MHC class II KO mice, which displayed similar defects in accumulation of K^b/OVA -specific $CD8^+$ T cells, and decreased IFN-

γ and TNF- α production (Fig. 3.S1F-3.S1H). These data show that, without CD4⁺ T cell help at priming, Ad vector-elicited CD8⁺ T cells undergo aberrant differentiation consisting of impaired effector phenotype, decreased cytotoxicity, and abnormal expression of key transcription factors.

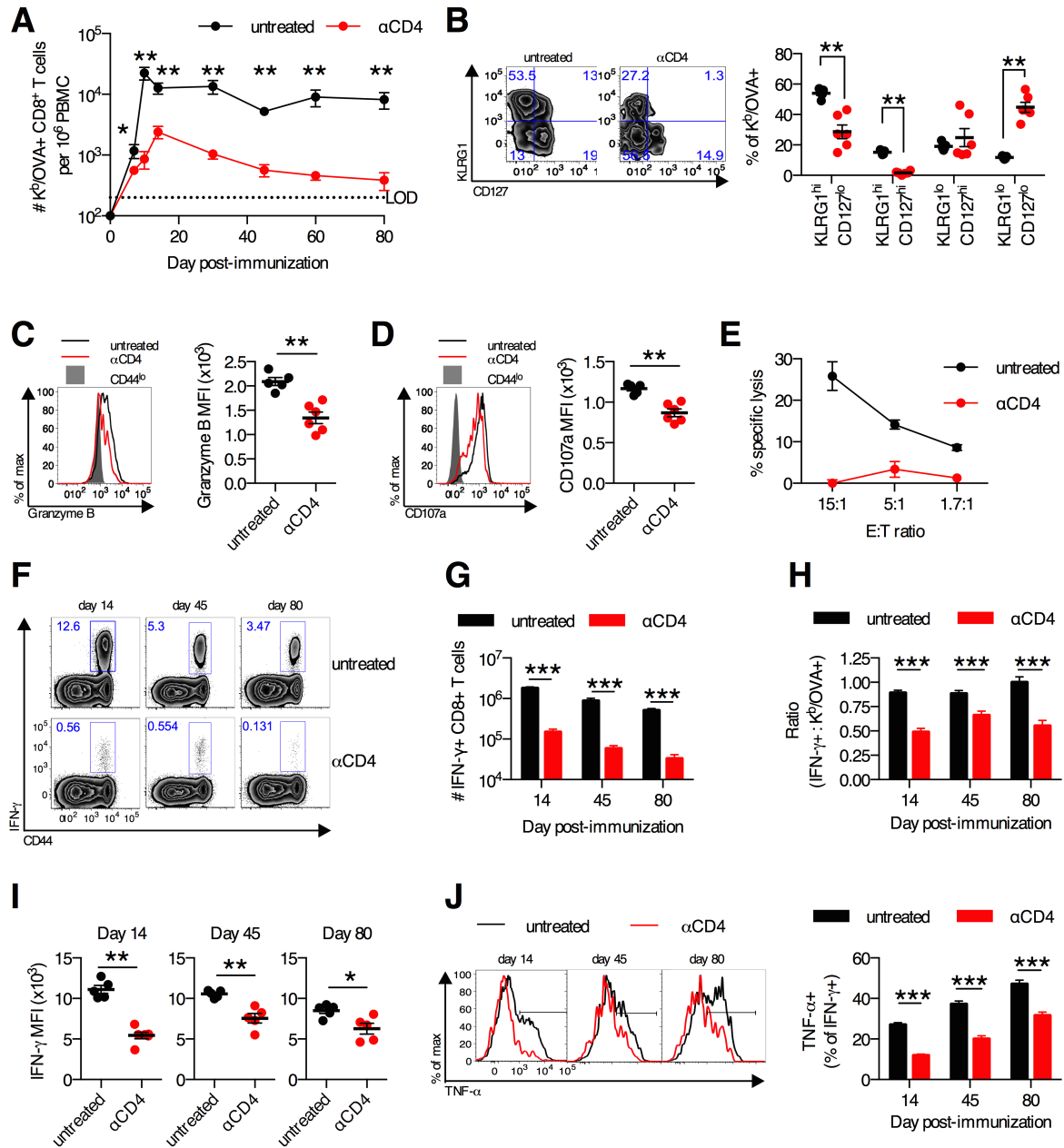


Figure 3.1. Impaired cytotoxicity and cytokine production by $CD8^+$ T cells primed in the absence of $CD4^+$ T cells. (A-J) C57BL/6 mice were treated with $\alpha CD4$ antibody or left untreated and immunized intramuscularly with 10^9 vp of Ad5-SIINFELK-Luc. (A) Frequency of K^b/OVA^+ $CD8^+$ T cells in blood. (B) KLRG1 and CD127 expression on K^b/OVA^+ $CD8^+$ T cells in the spleen on day 14 post-immunization. (C) Expression of Granzyme B on K^b/OVA^+ $CD8^+$ T cells in the spleen on day 14 post-immunization. (D) Expression of CD107a on IFN- γ^+ $CD8^+$ T cells following OVA₂₅₇₋₂₆₄ peptide stimulation of splenocytes from day 14 post-immunization. (E) 5 hr killing assay of OVA₂₅₇₋₂₆₄ peptide pulsed EL-4 cells by pooled splenic $CD8^+$ T cells at day 14 post-immunization. Each E:T ratio was performed in duplicate. *Continued* →

Figure 3.1 (Continued)

(**F**) Representative plots of IFN- γ^+ CD8 $^+$ T cells from the spleen. (**G**) Absolute frequency of IFN- γ^+ CD8 $^+$ T cells. (**H**) The fraction of OVA₂₅₇₋₂₆₄-specific CD8 $^+$ T cells that produce IFN- γ . (**I**) Mean fluorescence intensity (MFI) of IFN- γ^+ CD8 $^+$ T cells. (**J**) Representative plots of TNF- α production by IFN- γ^+ CD8 $^+$ T cells and group averages. Data are representative (**A-E, I**) of three experiments or pooled (**F-H, J**) from three experiments. N=5-6/group per experiment. Mean \pm SEM are shown. ***, P<0.001; **, P<0.01; *, P<0.05; Mann-Whitney U test.

CD8⁺ T cells primed without CD4⁺ T cell help express multiple inhibitory receptors.

To better understand how unhelped CD8⁺ T cells differentiated we also examined the expression of inhibitory receptors on antigen-specific CD8⁺ T cells following immunization of α CD4 treated mice. There was a substantial increase in the expression of PD-1 on K^b/OVA⁺ CD8⁺ T cells from α CD4 treated mice compared to untreated controls ($P < 0.01$; Fig. 3.2A). Increased expression of Tim-3, LAG-3, and 2B4 was also observed in α CD4 treated mice (Fig. 3.2A). In both untreated controls and α CD4 treated mice, PD-1 expression on K^b/OVA⁺ CD8⁺ T cells in the blood declined from day 7 to day 14 and then remained steady through day 80, and at all times PD-1 expression was significantly higher in α CD4 treated mice ($P < 0.05$; Fig. 3.2B). Long-term elevated PD-1 expression was also observed on K^b/OVA⁺ CD8⁺ T cells from CD4 KO and MHC class II KO mice (Fig. 3.S2A). Increased co-expression of multiple inhibitory receptors has been shown to correlate with reduced functionality of CD8⁺ T cells in the context of chronic viral infection (21). K^b/OVA⁺ CD8⁺ T cells from α CD4 treated mice exhibited significant increases ($P < 0.01$) in the fraction of cells that co-expressed 2, 3, or all 4 of the inhibitory receptors PD-1, Tim-3, LAG-3 and 2B4 compared to untreated controls (Fig. 3.2C). Thus, CD8⁺ T cells primed in the absence of CD4⁺ T cell help exhibit increased and prolonged expression of inhibitory receptors.

Increased expression of inhibitory receptors in the absence of CD4⁺ T cells is generalizable to multiple immunization regimens. Following Ad5HVR48-Gag vector immunization, unhelped CD8⁺ T cells specific for the immunodominant D^b/AL11 epitope recapitulated the dysfunction observed following Ad5-SIINFELK-Luc immunization (Fig. 3.S2B-3.S2C and data not shown). Following immunization with a replication-incompetent recombinant poxvirus vector (NYVAC-Env), Env-specific responses from α CD4 treated mice

were of reduced magnitude, had a less cytotoxic phenotype, and elevated PD-1 expression (Fig. 3.S2D-3.S2G). These data show that CD8⁺ T cell dysfunction following viral vector immunization in the absence of CD4⁺ T cells is generalizable to CD8⁺ T cells of multiple specificities, and to multiple replication-incompetent viral vector platforms.

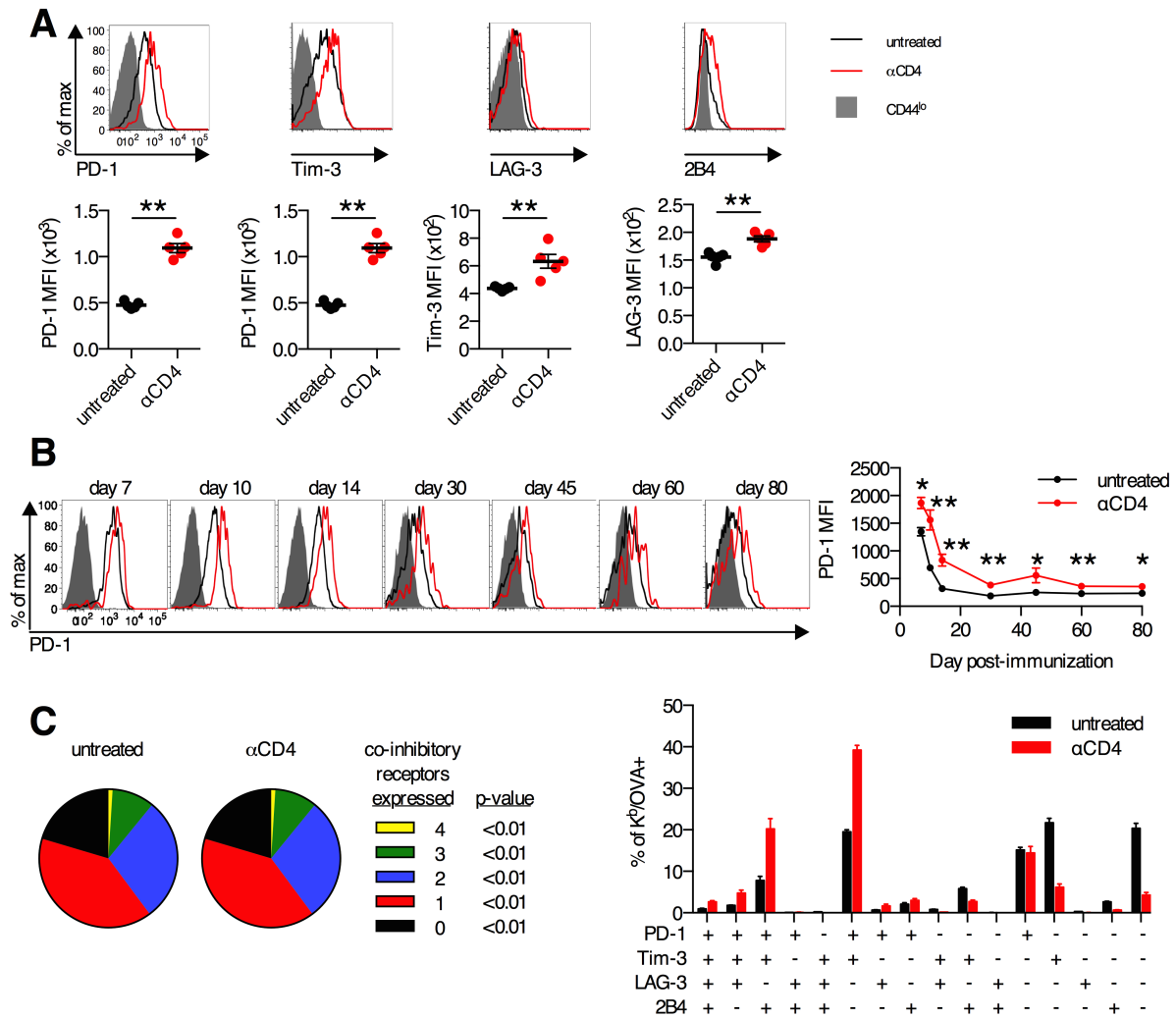


Figure 3.2. Elevated expression of inhibitory receptors on CD8⁺ T cells primed in the absence of CD4⁺ T cells. (A-D) C57BL/6 mice were treated with αCD4 antibody or left untreated and immunized intramuscularly with 10⁹ vp of Ad5-SIINFELK-Luc. (A) Expression of inhibitory receptors PD-1, Tim-3, LAG-3, and 2B4 on K^b/OVA⁺ CD8⁺ T cells in the spleen on day 14 post-immunization. (B) Longitudinal expression of PD-1 on K^b/OVA⁺ CD8⁺ T cells in the blood. (C) Co-expression of inhibitory receptors PD-1, Tim-3, LAG-3, and 2B4 on K^b/OVA⁺ CD8⁺ T cells in the spleen on day 14 post-immunization, either as number of inhibitory receptors expressed (top) or specific combinations (bottom). Data are representative of three experiments (n=5-6/group per experiment). Mean ± SEM are shown. **, P<0.01; *, P<0.05; Mann-Whitney U test.

Absence of CD4⁺ T cells induces early transcriptional signatures of T cell exhaustion and anergy in CD8⁺ T cells.

Given the multifaceted abnormalities in CD8⁺ T cells primed without CD4⁺ T cell help, we evaluated the transcriptional profile of these cells. On day 14 post-immunization with Ad5HVR48-Gag, D^b/AL11⁺ CD8⁺ T cells from the spleen of untreated control or α CD4 treated mice were sorted and gene expression profiling was performed. Highly upregulated genes in D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice compared to untreated controls were *Havcr2* (encodes Tim-3), *Pdcd1* (encodes PD-1), *Cd244* (encodes 2B4), *Lag3* (encodes LAG-3), and *Ctla4* (encodes CTLA4) (Fig. 3.3A). In contrast, highly upregulated genes in D^b/AL11⁺ CD8⁺ T cells from untreated control mice compared to α CD4 treated were *Klrg1* (encodes KLRG1), *Gzmm* (encodes Granzyme M), and *Gzma* (encodes Granzyme A) (Fig. 3.3A). These results highlight the over-expression of inhibitory markers by CD8⁺ T cells as a key difference in the absence of CD4⁺ T cell help.

To further probe the concerted transcriptional differences between CD8⁺ T cells primed with and without CD4⁺ T cell help we utilized gene set enrichment analysis (GSEA) (22). GSEA analysis identified multiple cell cycle pathways that were down-regulated in D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice (Fig. 3.3B). Consistent with this, reduced proliferation of D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice was confirmed experimentally (Fig. 3.S3A). Conversely, multiple T cell receptor (TCR) signaling, cytokine signaling (notably STAT5), and cell death pathways were enriched in D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice (Fig. 3.3B). Increased apoptosis of D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice was also confirmed experimentally (Fig. 3.S3B). Together the decreased proliferation and increased apoptosis of unhelped D^b/AL11⁺ CD8⁺ T cells explains their reduced frequencies.

The molecular signatures of CD8⁺ T cell exhaustion from two previous studies (23, 24) were significantly enriched in D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice (Fig. 3.3C). The converse effector gene signature from each study was significantly enriched in D^b/AL11⁺ CD8⁺ T cells from untreated control animals (Fig. 3.S3C). A molecular signature of T cell anergy was also significantly enriched in D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice (Fig. 3.3C). Leading edge analysis of enriched genes from the exhaustion and anergy signatures identified that these two signatures were largely non-overlapping, which suggests the simultaneous expression of two distinct transcriptional programs in these cells (Fig. 3.3D and Fig. 3.S3D). Expression of a T cell anergy signature was not simply due to absence of signaling via CD28, as an antigen-specific CD8⁺ T cell response was completely absent in CD80/CD86 double KO mice, and thus did not recapitulate the absence of CD4⁺ T cells (Fig. 3.S3E). The transcriptional signature of exhaustion in CD8⁺ T cells primed without CD4⁺ T cell help is consistent with the elevated expression of inhibitory receptors and hypofunctionality, and the transcriptional signature of anergy is consistent with the apparent rapidity of this dysfunction.

A recent report has demonstrated that the transcription factor NFAT when acting independently of AP-1 can induce expression of exhaustion- and anergy-associated genes (25). NFATc1 was substantially over-expressed in D^b/AL11⁺ CD8⁺ T cells from α CD4 treated mice (Fig. 3.3A), and these cells were enriched for signatures of NFAT signaling (Fig. 3.3B). We thus hypothesized that AP-1-independent NFAT signaling (here termed NFAT Δ AP-1) was driving the dual expression of exhaustion and anergy transcriptional signatures. Consistent with this hypothesis, the NFAT Δ AP-1 gene signature was enriched in cells from α CD4 treated mice, as were the specific subsets of genes previously shown to be regulated by NFAT Δ AP-1 and involved in anergy or exhaustion (Fig. 3.3E-3.3F). An additional signature for non-canonical

NFAT signaling was also significantly enriched (Fig. 3.3E-3.3F). Of note, no AP-1 target pathways were enriched in cells from α CD4 treated mice (data not shown). These data are consistent with a model where atypical NFAT signaling in antigen-specific CD8⁺ T cells from α CD4 treated mice appears to drive, in part, the simultaneous expression of exhaustion and anergy gene signatures in these cells.

Provision of CD4⁺ T cell help after immunization fails to rescue the dysfunctional phenotype of CD8⁺ T cells primed in the absence of CD4⁺ T cells.

Chronic viral infection induces classical CD8⁺ T cell exhaustion that becomes progressively more profound over time (26-28). In contrast, anergic T cells rapidly display functional defects (29). Thus, we sought to determine if the defects of CD8⁺ T cells primed without CD4⁺ T cell help were irreversibly imprinted at priming, or if the differentiation was a progressive process. We experimentally reconstituted CD4⁺ T cell help after immunization to explore whether this could rescue the dysfunctional state of CD8⁺ T cells primed in the absence of CD4 T cells. A recently described experimental system was utilized to generate mice permanently lacking endogenous CD4⁺ T cells (9, 30). CD45.1⁺ congenic mice underwent adult thymectomy and were treated with α CD4 Ab or left untreated as a control (Fig. 3.4A). One month post- α CD4 Ab treatment, all α CD4 Ab had been cleared from the serum (data not shown). To correct for the lack thymic output, 5×10^2 naïve OT-I CD8⁺ T cells (specific for the K^b/OVA₂₅₇₋₂₆₄ epitope) were adoptively transferred into all animals. Finally, mice either received no adoptively transferred CD4⁺ T cells or 5×10^4 naïve OT-II CD4⁺ T cells (specific for the I-A^b/OVA₃₂₃₋₃₃₉ epitope) on day -1, 3, 7, or 10 post-immunization (Fig. 3.4A).

Mice that received α CD4 antibody and did not receive adoptive transfer of CD4⁺ T cells had OT-I CD8⁺ T cell responses of a reduced magnitude, and the responding cells expressed less KLRG1 and more PD-1 than OT-I cells from control mice that did not receive α CD4 antibody (Fig. 3.4B-4D, red lines). These responses recapitulated those seen when endogenous K^b/OVA-specific CD8⁺ T cells are measured, which validates the use of the CD8⁺ T cell adoptive transfer system. Adoptive transfer of naïve OT-II CD4⁺ T cells prior to immunization resulted in expansion and differentiation of OT-I CD8⁺ T cells that was largely equivalent to the responses

seen in mice that had not been treated with α CD4 antibody (black versus blue lines; Fig. 3.4B-3.4D). Thus, adoptive transfer of naïve OT-II CD4⁺ T cells recapitulates the CD4⁺ T cell help provided by endogenous CD4⁺ T cells.

When naïve OT-II CD4⁺ T cells were transferred into mice on day 3 post-immunization, the expansion of the OT-I CD8⁺ T cell responses was partially rescued so that at day 14 post-immunization the responses were equivalent to the control group (purple line; Fig. 3.4B), but the peak of the response was delayed and of substantially reduced magnitude compared to the control groups. Adoptive transfer of naïve OT-II CD4⁺ T cells on day 7 or 10 post-immunization had no detectable impact on the magnitude of the OT-I CD8⁺ T cell responses (Fig. 3.4B). Despite the modest effect on the magnitude of the CD8⁺ T cell response, adoptive transfer of naïve OT-II CD4⁺ T cells on day 3 post-immunization did not rescue the expression of KLRG1 or decrease the expression of PD-1 relative to mice that did not receive adoptive transfer of CD4⁺ T cells (purple versus red lines; Fig. 3.4C and 3.4D). Concordant with these data, adoptive transfer of naïve OT-II CD4⁺ T cells on day 7 or 10 post-immunization also had no impact on the phenotype of the CD8⁺ T cells (green and grey lines; Fig. 3.4C and 3.4D). These data suggest that CD4⁺ T cells are essential during priming to provide help to responding CD8⁺ T cells, and the absence of help during this period results in irreversible dysfunction.

Persistent antigen is a critical driving factor in chronic infection-induced exhaustion (31-33). However, given the immediate irreversible nature of the dysfunction we observed in CD8⁺ T cells primed without CD4⁺ T cell help, we hypothesized that persistent antigen would not be a major causative factor in our system. To test this, we utilized a previously described Ad vector in which the transgene is under the control of a tetracycline repressive element (Ad5-tTA-SIINFEKL-Luc) (34). Despite robust transgene silencing, no alteration in the frequency,

phenotype or functionality of antigen-specific CD8⁺ T cells primed without CD4⁺ T cells was observed (Fig. 3.S4). Together these data suggest that while immediate CD8⁺ T cell dysfunction shares functional, transcriptional, and phenotypic characteristics with chronic infection-induced exhaustion, one of the major causative pathways does not appear to be shared.

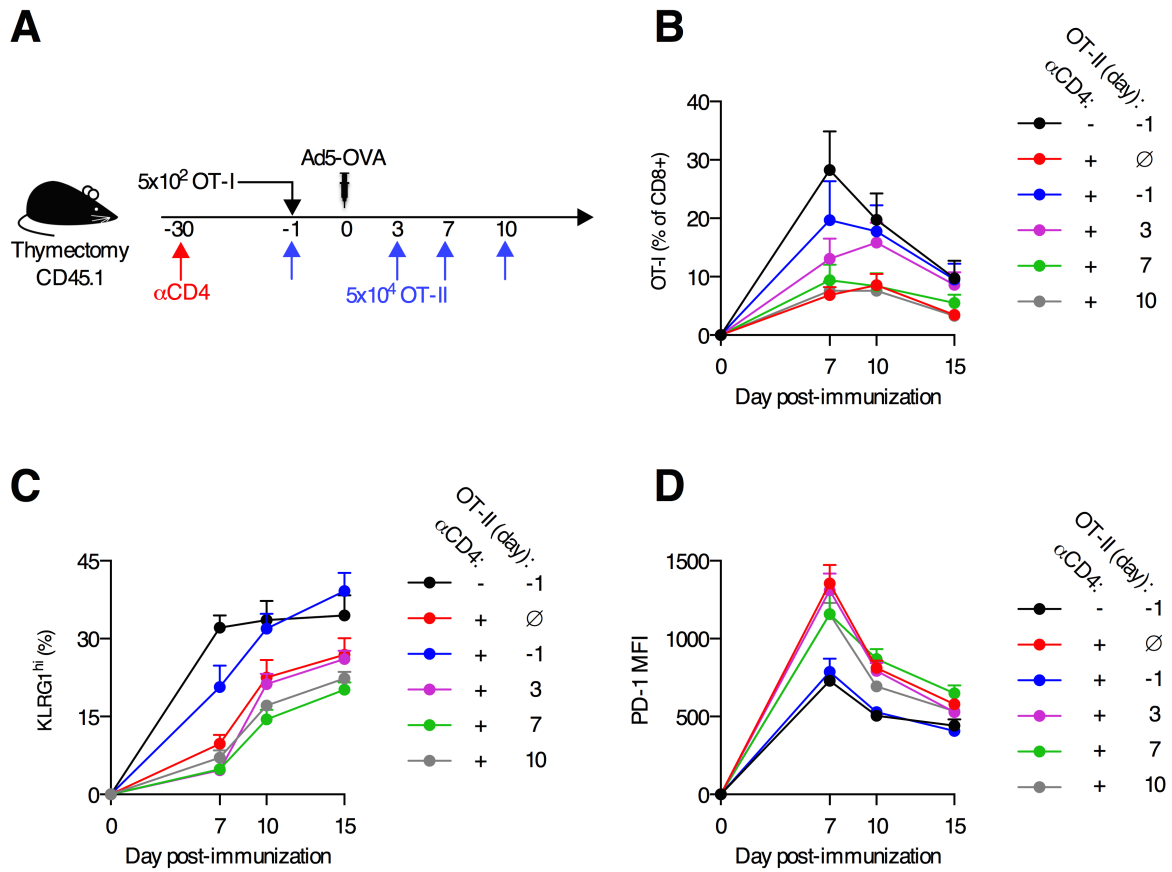


Figure 3.4. Provision of CD4⁺ T cell help after immunization does not rescue the dysfunctional phenotype of CD8⁺ T cells primed without CD4⁺ T cell help. (A-D) Experimental schematic. CD45.1⁺ B6 mice underwent adult thymectomy and were treated with α CD4 antibody or left untreated 30 days prior to immunization. 5×10^2 naïve CD45.2⁺ OT-I CD8⁺ T cells were adoptively transferred one day prior to immunization. Mice were immunized intramuscularly with 10^9 vp of Ad5-OVA. 5×10^4 naïve CD45.2⁺ OT-II CD4⁺ T cells were adoptively transferred on day -1, 3, 7, or 10 post-immunization. For one control group no OT-II CD4⁺ T cells were transferred. **(B)** Frequency of OT-I CD8⁺ T cells in the blood. **(C)** Fraction of blood OT-I CD8⁺ T cells expressing KLRG1. **(D)** PD-1 expression on blood OT-I CD8⁺ T cells. Data are pooled from two experiments **(B, C)** or representative of two experiments **(D)**. N=4-5/group per experiment. Mean + SEM are shown.

Blockade of PD-1 signaling during priming partially rescues the CD8⁺ T cell response generated without CD4⁺ T cell help.

Given the elevated expression of PD-1 on CD8⁺ T cells primed without CD4⁺ T cell help, we hypothesized that blockade of PD-1 might rescue the differentiation of these cells, as has been reported for chronic infections or cancer (35-39). To test this hypothesis, mice were depleted of CD4⁺ T cells or left untreated, immunized with Ad5-SIINFEKL-Luc, and treated with α PD-L1 antibody or an isotype control on day -1, 2, 5, and 8 post-immunization (Fig. 3.5). Blockade of PD-1 signaling (via α PD-L1 antibody) led to a partial rescue (6-fold; $P < 0.01$) in the accumulation of K^b/OVA⁺ CD8⁺ T cells primed in the absence of CD4⁺ T cell help (Fig. 3.5A). Administration of α PD-L1 antibody significantly increased the fraction of K^b/OVA⁺ CD8⁺ T cells that expressed KLRG1 and significantly decreased the fraction of cells that were in an undifferentiated KLRG1^{lo}CD127^{lo} state ($P < 0.05$; Fig. 3.5B). Administration of α PD-L1 antibody following Ad5-HVR48-Gag immunization of α CD4 treated mice also partially rescued the frequency and KLRG1 expression of D^b/AL11⁺ CD8⁺ T cells (data not shown). α PD-L1 antibody treatment rescued the defect in Granzyme B expression observed in K^b/OVA⁺ CD8⁺ T cells primed in the absence of CD4⁺ T cells ($P < 0.01$; Fig. 3.5C). Phenotypic rescue of K^b/OVA⁺ CD8⁺ T cells by α PD-L1 antibody treatment also corresponded to a substantial increase in cytotoxicity as measured using an *ex vivo* killing assay (Fig. 3.5D). The fraction and number of IFN- γ ⁺ CD8⁺ T cells was significantly increased in α PD-L1 antibody treated mice compared to isotype controls ($P < 0.01$; Fig. 3.5E and 3.5F). Finally, α PD-L1 treatment lead to a partial rescue in the ability of IFN- γ ⁺ CD8⁺ T cells to co-produce TNF- α compared to mice that received an isotype control antibody ($P < 0.01$; Fig. 3.5E and 3.5F). To confirm these findings we also utilized an α PD-1 antibody to block receptor ligation. Treatment with an α PD-1 antibody recapitulated the

effect of treatment with an α PD-L1 antibody for all parameters analyzed (Fig. 3.S5). Collectively, these data demonstrate signaling via PD-1 early post-immunization plays a critical role in modulating the dysfunction observed in CD8⁺ T cells primed without CD4⁺ T cell help.

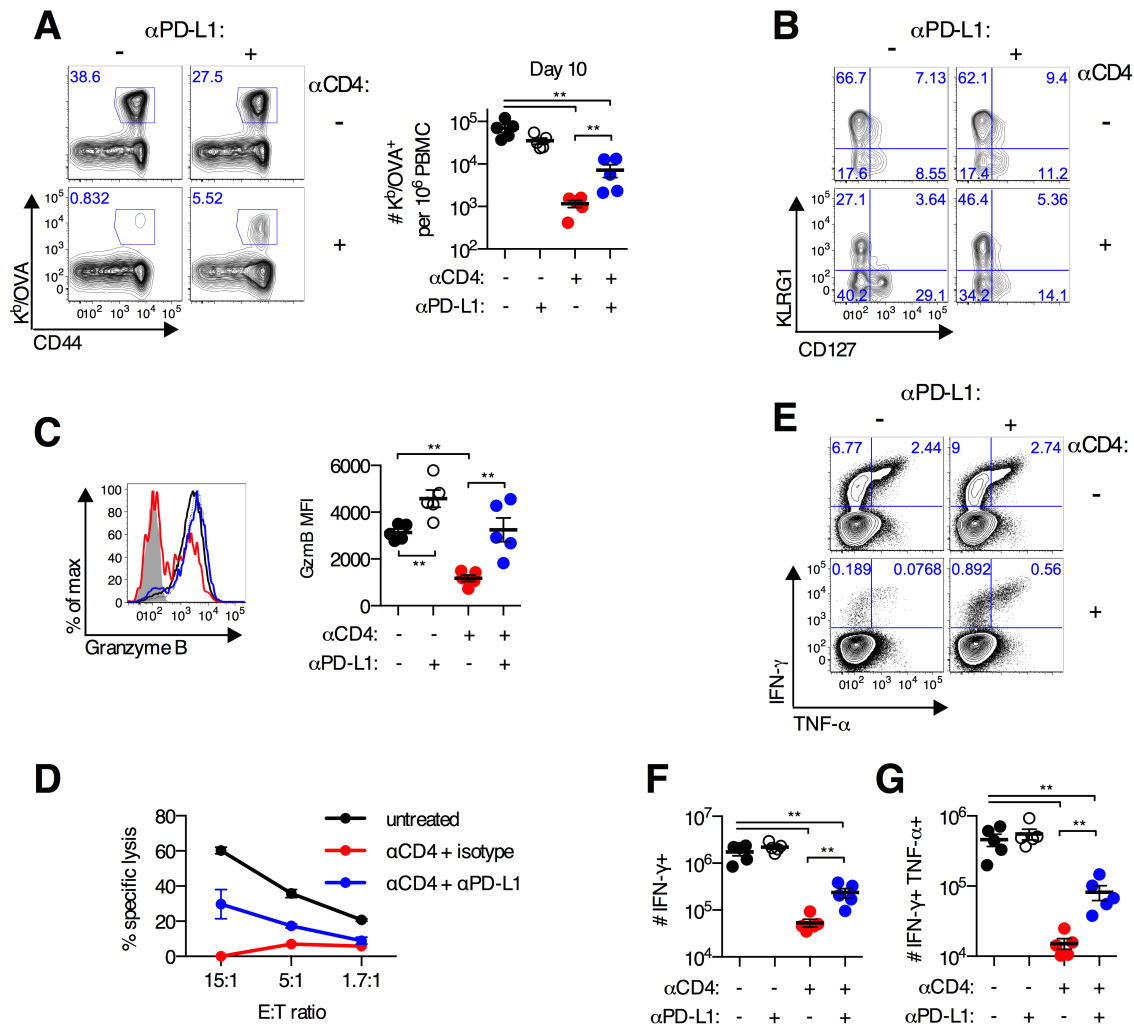


Figure 3.5. $\alpha PD-L1$ antibody treatment partially rescues differentiation of $CD8^+$ T cells primed in the absence of $CD4^+$ T cells. (A-G) C57BL/6 mice were treated with $\alpha CD4$ antibody or left untreated and immunized intramuscularly with 10^9 vp of Ad5-SIINFEKL-Luc. Mice were administered $\alpha PD-L1$ or isotype control antibody every 3 days beginning one day prior to immunization. (A) Representative plots and absolute number of K^b/OVA^+ $CD8^+$ T cells in the blood on day 10 post-immunization. (B) KLRG1 and CD127 expression on K^b/OVA^+ $CD8^+$ T cells in the blood on day 10 post-immunization. (C) Expression of Granzyme B on K^b/OVA^+ $CD8^+$ T cells in the blood on day 10 post-immunization. (D) 5 hour killing assay of $OVA_{257-264}$ peptide pulsed EL-4 cells by pooled $CD8^+$ T cells from day 10 post-immunization. E:T ratio was performed in duplicate for untreated and $\alpha CD4 + \alpha PD-L1$ and singlet for $\alpha CD4 + isotype$. (E) Representative plots of IFN- γ and TNF- α by $CD8^+$ T cells from the spleen upon *ex vivo* stimulation with $OVA_{257-264}$ peptide. (F) Absolute number of $OVA_{257-264}$ -specific $CD8^+$ T cells that produce IFN- γ . (G) Absolute number of $OVA_{257-264}$ -specific $CD8^+$ T cells that co-produce IFN- γ and TNF- α . Data are representative of two to four experiments per group (n=5-8/group per experiment). Mean \pm SEM are shown. **, P<0.01; *, P<0.05; Mann-Whitney U test.

Perturbed IL-2 signaling in the absence of CD4⁺ T cells is involved in CD8⁺ T cell dysfunction.

We sought to determine if IL-2 signaling was perturbed in CD8⁺ T cells primed without CD4⁺ T cell help, as IL-2 signaling has a well established role in T cell anergy (40), and a more recently described role in CD8⁺ T cell exhaustion (41). Ova-specific CD8⁺ T cells were first detectable in the draining LNs on day 4 post-immunization (Fig. 3.S6A-3.S6C), consistent with a previous report (42). Strikingly, K^b/OVA⁺ CD8⁺ T cells from α CD4 treated mice already had a significant reduction in expression of CD25 (high affinity IL2R α receptor) compared to untreated controls on day 4 ($P < 0.01$; Fig. 3.6A). Intriguingly, several other genes that are known to be regulated by IL-2 signaling, including up-regulation of CD71, CD98, and Granzyme B, as well as increased cell size and granularity during proliferation (43-45), were all perturbed in the absence of CD4⁺ T cells (Fig. 3.1 and Fig. 3.S6D-3.S6E). No difference in expression of co-stimulatory receptors was observed (Fig. 3.S6D-3.S6E). These data suggest that in the absence of CD4⁺ T cell help there are alterations in IL-2 signaling to CD8⁺ T cells.

We thus hypothesized that administration of rIL-2 might correct the dysfunctional differentiation of CD8⁺ T cells in the absence of CD4⁺ T cell help. Administration of rIL-2 to α CD4 antibody treated mice, but not untreated controls, increased the frequency of K^b/OVA⁺ CD8⁺ T cells by 8.6-fold and the number by 7-fold on day 10 post-immunization ($P < 0.01$; Fig. 3.6B). Administration of rIL-2 significantly increased Granzyme B expression ($P < 0.01$; Fig. 3.6C) and also corrected the defect in differentiation to a KLRG1^{hi}CD127^{lo} effector phenotype (Fig. 3.6D). Administration of rIL-2 decreased PD-1 expression on K^b/OVA⁺ CD8⁺ T cells from α CD4 treated mice to levels seen on K^b/OVA⁺ CD8⁺ T cells from untreated control animals ($P < 0.01$; Fig. 3.6E). However, co-administration of rIL-2 and α PD-L1 antibody did not

significantly increase the frequency of K^b/OVA^+ $CD8^+$ T cells in the blood compared to either treatment alone (Fig. 3.6E). Co-administration of rIL-2 and $\alpha PD-L1$ antibody also did not impact on PD-1, KLRG1, or Granzyme B expression any more than one treatment alone (data not shown). Taken together, repeated administration of low dose rIL-2 can partially rescue the impaired accumulation and effector differentiation of K^b/OVA^+ $CD8^+$ T cells in $\alpha CD4$ treated mice but appears to do so in a redundant manner to blockade of PD-1 signaling. These data suggest that administration of rIL-2 is sufficient to properly differentiate unhelped $CD8^+$ T cells to a state where they are no longer responsive to blockade of PD-1 signaling.

Given these findings, we sought to determine if absence of IL-2 signaling on $CD8^+$ T cells was sufficient to recapitulate the absence of $CD4^+$ T cell help. Naïve wild type and $CD25$ KO OT-I $CD8^+$ T cells were transferred at a 1:1 ratio into the same recipient (Fig. 3.6G). On day 10 post-immunization, $CD25$ KO OT-I $CD8^+$ T cells displayed a massive defect in frequency compared to their wild type counterparts ($P < 0.01$; Fig. 3.6H), and these responding cells have reduced expression of Granzyme B ($P < 0.01$; Fig. 3.6I). However, $CD25$ KO OT-I $CD8^+$ T cells had unimpaired upregulation of KLRG1 (Fig. 3.6J) and did not over-express PD-1 relative to wild type OT-I $CD8^+$ T cells (Fig. 3.6K). Thus, absence of cell-intrinsic high-affinity IL-2 signaling by $CD8^+$ T cells is sufficient to recapitulate the reduced frequency and reduced effector functionality of $CD8^+$ T cells when $CD4^+$ T cells are absent. However, the absence of high-affinity IL-2 signaling is insufficient to fully recapitulate the dysfunction observed in the absence of $CD4^+$ T cells, and suggests that $CD4^+$ T cells provide help by multiple mechanisms. The observation that rIL-2 is sufficient to suppress PD-1 expression (Fig. 3.6E), but absence of IL-2 signaling is not sufficient to induce PD-1 over-expression (Fig. 3.6K) supports the conclusion that $CD4^+$ T cell help involves multiple mechanisms.

Figure 3.6. Early IL-2 treatment rescues proliferation and effector differentiation of CD8⁺ T cells primed in the absence of CD4⁺ T cells. (A) Expression of CD25 on K^b/OVA⁺ CD8⁺ T cells in the iliac LNs. (B-E) C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10⁹ vp of Ad5-SIINFELK-Luc. Mice were administered with recombinant mouse IL-2 (rIL-2) or PBS control twice daily from day 3 to 10 post-immunization. (C) Frequency of K^b/OVA⁺ CD8⁺ T cells in blood on day 10 post-immunization. (D) Expression of PD-1 on K^b/OVA⁺ CD8⁺ T cells in the blood. (E) KLRG1 and CD127 expression on K^b/OVA⁺ CD8⁺ T cells in the blood. (F) Expression of Granzyme B on K^b/OVA⁺ CD8⁺ T cells in the blood. (G-K) Mice were administered with recombinant mouse IL-2 (rIL-2) or PBS control twice daily from day 3 to 10 post-immunization and α PD-L1 antibody or isotype control once every three days beginning on day 0. Frequency of K^b/OVA⁺ CD8⁺ T cells in blood on day 10 post-immunization. (G-K) WT and CD25 KO OT-I CD8⁺ T cells were mixed at a 1:1 ratio and adoptively transferred into congenically marked mice one day prior to immunization. Mice were immunized intramuscularly with 10⁹ vp of Ad5-SIINFELK-Luc. Responses were assessed on day 10 for frequency of OT-I cells in the blood (H), Granzyme B expression (I), KLRG1 expression (J), and PD-1 expression (K) on OT-I CD8⁺ T cells. Data are representative of two experiments (n=4-5/group per experiment). Mean \pm SEM are shown. **, P<0.01; *, P<0.05; Mann-Whitney U test. *Figure on next page* →

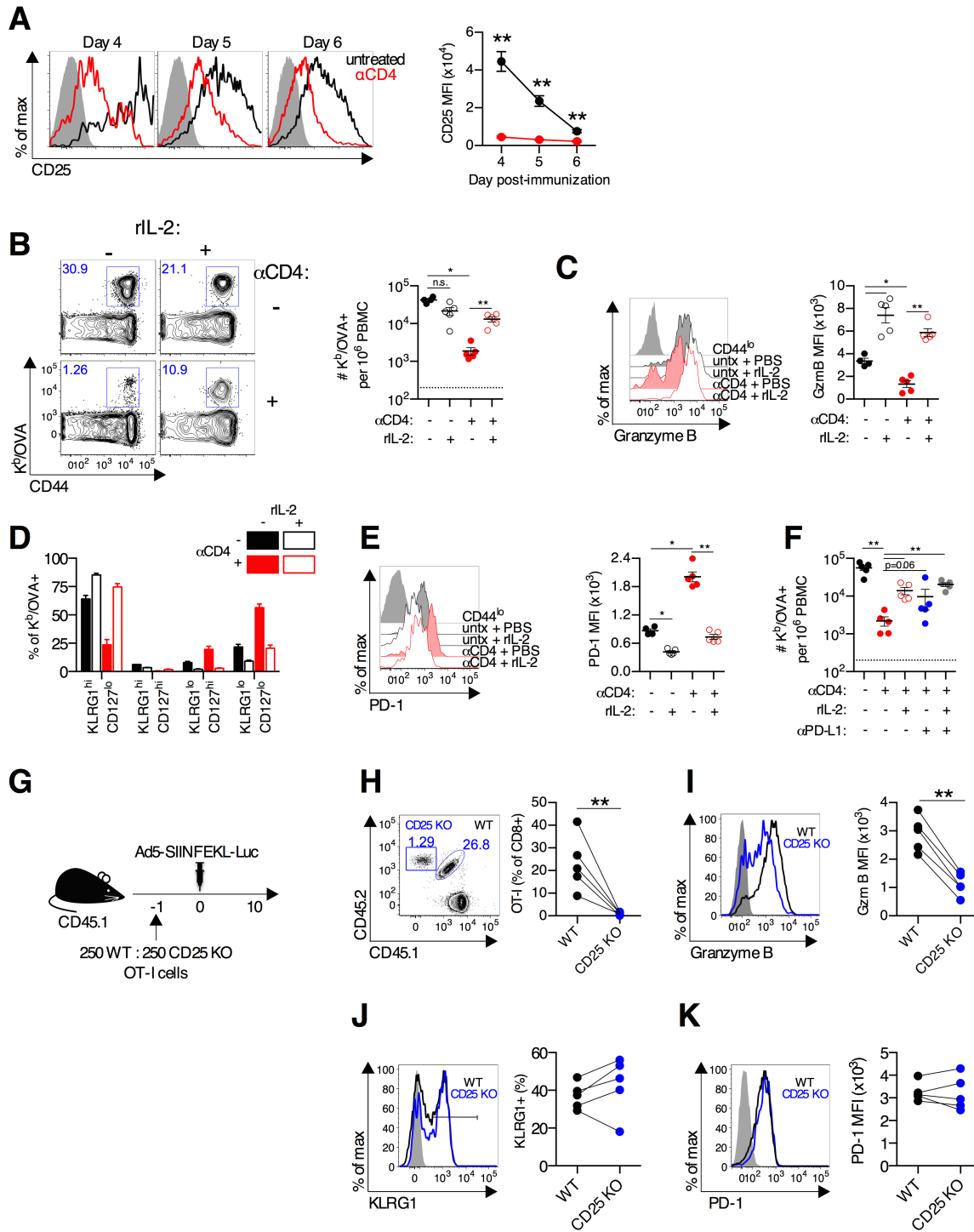


Figure 3.6 (Continued)

DISCUSSION

CD4⁺ T cell help has been shown to be critical in many settings for the optimal expansion of CD8⁺ T cell responses (46). However, the role of CD4⁺ T cells in the immediate phenotypic imprinting of CD8⁺ T cells remains poorly understood. Our data demonstrate a novel means by which CD4⁺ T cells provide help following Ad vector immunization to prevent early exhaustion-like dysfunction of vaccine-elicited CD8 T cells. Following Ad vector immunization of CD4⁺ T cell deficient mice, the CD8⁺ T cell responses are of reduced frequency, have reduced cytotoxicity and cytokine production, and exhibit an aberrant phenotype characterized by elevated expression of multiple inhibitory receptors. CD8⁺ T cells elicited without CD4⁺ T cell help expressed transcriptional signatures of CD8⁺ T cell exhaustion and T cell anergy, which appears to be driven, in part, by excessive AP-1-independent NFAT signaling when CD4⁺ T cells were absent. Anergy and exhaustion are largely distinct differentiation states (47), and thus the underlying dysfunction of these CD8⁺ T cells appears to be caused by simultaneous expression of multiple inhibitory transcriptional programs. Irreversible abnormalities in CD8⁺ T cell differentiation occurred within days of immunization when CD4⁺ T cells were absent. This dysfunctional differentiation is a multi-faceted process, but we have identified key roles for elevated PD-1 and reduced IL-2 signaling. Collectively, this study identifies a previously unappreciated role of CD4⁺ T cells in immediate programming of CD8⁺ T cell effector phenotype, by modulating coordinated activation of key T cell-specific transcription factors, including AP-1.

Previous studies have shown that CD4⁺ T cells are critical for driving optimal primary CD8⁺ T cell expansion in several experimental systems (2, 3, 5, 9, 16, 48). In this study, we demonstrate that CD4⁺ T cells are required at priming for optimal induction of CD8⁺ T cells by

Ad vector immunization, consistent with previous reports (11-14). In addition to modulating CD8⁺ T cell expansion, we identify a critical role for CD4⁺ T cells in driving acquisition of CD8⁺ T cell effector functions, which is in contrast to previous reports that, depending on the experimental setup, determined CD4⁺ T cells either had no role in acquisition of effector phenotype (16) or restrained effector differentiation (4, 5). We have thus identified CD4⁺ T cell-derived signals can be crucial for the acquisition of effector function and phenotype by CD8⁺ T cells.

Our data show that CD4⁺ T cell help is required at priming to ensure proper effector CD8⁺ T cell differentiation in this system. Provision of CD4⁺ T cell help on day 3 after vaccination can partially rescue the accumulation of antigen-specific CD8⁺ T cells, but it does not correct the phenotypic dysfunction of CD8⁺ T cells (Fig. 3.4). We have recently demonstrated that the presence of CD4⁺ T cells is required for several days post-immunization for proper expansion and effector differentiation of CD8⁺ T cells following Ad vector immunization (11). In that work, the earlier depletion of CD4⁺ T cells occurred the more profoundly impaired the CD8⁺ T cell responses were. Synthesizing these studies demonstrates that CD4⁺ T cells provide indispensable signals during priming and then continue to provide signals for a week post-immunization to maintain CD8⁺ T cell proliferation and reinforce the established effector differentiation pathway.

CD8⁺ T cells primed without CD4⁺ T cell help display an altered phenotype with elevated expression of inhibitory receptors, decreased *ex vivo* cytotoxicity, decreased ability to produce cytokines, and a transcriptional signature of exhaustion (Fig. 3.1-3.3), which are all characteristics indicative of exhausted CD8⁺ T cells (21, 23, 24, 26, 35, 49-51). Blockade of PD-1 signaling partially rescued responses in our system (Fig. 3.5), as did administration of rIL-2

(Fig. 3.6), and both of these treatments rescue exhausted CD8⁺ T cells (35, 41). However, CD8⁺ T cell exhaustion involves a strong temporal component where CD8⁺ T cells become progressively more impaired the longer the cells remain in an environment of persistent viremia (26-28, 52, 53). By contrast, in our model, CD8⁺ T cell dysfunction occurs within days of immunization (Fig. 3.4). Consistent with this, experimental removal of persistent expression of antigen in mice depleted of CD4⁺ T cells did not alter the functionality or phenotype of these dysfunctional CD8⁺ T cells (Fig. 3.S4). In these studies, we describe a novel aberrant CD8⁺ T cell differentiation state that is phenotypically and functionally akin to exhaustion, but occurs immediately upon priming.

When CD8⁺ T cells fail to receive the complete array of priming signals they immediately enter a dysfunctional state termed “split-anergy” (54). This split-anergy is characterized by reduced proliferative capacity, but no defects in IFN- γ production or cytotoxic function are observed (55). While the CD8⁺ T cell dysfunction observed in our study occurs immediately upon priming and these cells express a transcriptional program of anergy, the induced cells have dramatic impairments in IFN- γ production and cytotoxic function, and thus are not functionally similar to cells in a state of split-anergy. However, the dysfunctional CD8⁺ T cells we describe and split-anergy CD8⁺ T cells are responsive to reinvigoration by rIL-2 administration (Fig. 3.6 and (40), respectively). However, following Ad vector immunization, IL-2 was required to endow CD8⁺ T cell responses with proliferative capacity and effector functionality (Fig. 3.6). By contrast, IL-2 signaling to CD8⁺ T cells in a state of split-anergy serves primarily to drive proliferation (40) as these cells maintain robust effector functionality (55, 56). Thus, while these unhelped CD8⁺ T cells have characteristics of anergy, the

functionality of these cells is distinct from the previously characterized state of CD8⁺ T cell anergy.

It has recently been demonstrated that expression of an engineered NFAT that is incapable of interacting with AP-1 is sufficient to drive expression of anergy and exhaustion-related genes (25). Elevated NFAT, but not AP-1 family proteins, were enriched in CD8⁺ T cells from CD4⁺ T cell deficient mice, and gene signatures of NFAT signaling were significantly enriched (Fig. 3.3). Specifically, the gene signature of AP-1-independent NFAT signaling was enriched as were the subset of NFAT-regulated genes associated with anergy and exhaustion. Thus, we propose a model whereby a major driving factor of the observed CD8⁺ T cell dysfunction is due to elevated non-canonical NFAT signaling. The partial prevention of this phenotype by rIL-2 administration suggests that, in the context of Ad vector immunization, CD4⁺ T cell-derived IL-2 is responsible for inducing AP-1 signaling on CD8⁺ T cells, as has been reported in an *in vitro* model of CD4⁺ T cell anergy (57). Future experiments will be required to test this hypothesis. Additionally, given the clear data that manipulation of IL-2 signaling can only partially rescue or induce CD8⁺ T cell dysfunction, additional mechanisms of CD4⁺ T cell help remain to be identified.

In conclusion, we demonstrate that CD4⁺ T cells are required immediately upon antigen priming for the optimal expansion and effector differentiation of viral vector vaccine induced CD8⁺ T cells. The absence of CD4⁺ T cell help results in a multi-faceted and immediate dysfunction of responding CD8⁺ T cells that functionally mirrors many of the characteristics of T cell exhaustion. These dysfunctional cells express both exhaustion and anergy gene signatures, which appears to be due to increased AP-1-independent NFAT signaling. This dysfunction is imprinted, in part, by elevated PD-1 signaling and reduced IL-2 signaling. These data suggest a

novel mechanism of immediate CD4⁺ T cell help to prime effector CD8⁺ T cell function. Furthermore, these findings suggest a potential means of improving the immunogenicity of viral vector immunization in the context of reduced CD4⁺ T cell functionality by manipulation of these pathways.

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**Chapter 4: Transient CD4⁺ T Cell Depletion Results in the Delayed Development of
Functional Vaccine-Elicited Antibody Responses**

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ABSTRACT

Replication-incompetent adenovirus (Ad) vectors are used in prime-boost immunization regimens to induce robust antigen-specific antibody responses against their expressed transgene antigens, but how Ad vector-elicited antibody responses are regulated remains unknown. Here we demonstrate that induction of antibody responses can occur at a time displaced from the time of Ad vector immunization by depletion of CD4⁺ T cells. Transient depletion of CD4⁺ T cells at the time of immunization delayed the development of antigen-specific antibody responses but did not permanently impair their development or induce tolerance against the encoded transgene. Upon CD4⁺ T cell recovery, transgene-specific serum IgG antibody titers developed and reached a concentration equivalent to undepleted control animals. These delayed antibody responses exhibited no functional defects with regards to isotype, functional avidity, expansion after boosting immunization, or their capacity to neutralize an SIV Env-expressing pseudovirus. The development of these delayed transgene-specific antibody responses requires thymus-mediated reconstitution of the CD4⁺ T cell population, and involves the *de novo* generation of germinal center responses. These data demonstrate the ability to modulate the timing of vaccine-elicited antibody responses by manipulation of CD4⁺ T cells and may have important implications for the development of vaccines designed to elicit antibody responses.

INTRODUCTION

Replication-incompetent recombinant adenovirus (Ad) vectors are a promising class of candidate vaccines for several diseases of major public health importance including human immunodeficiency virus-1 (HIV-1) (1, 2), hepatitis C (1-4), ebola (5, 6), tuberculosis (7), and malaria (8). Ad vectors have primarily been pursued as vaccine platforms due to their ability to induce strong CD8⁺ T cell responses (3, 5, 7, 9), but they can also induce robust transgene antigen-specific antibody responses (10-12). In a preclinical trial of Ad vector-based HIV-1 vaccines, Ad vector expressing SIV Env prime followed by a soluble SIV Env protein boost resulted in protection of 50% of rhesus monkeys challenged with a difficult-to-neutralize virus, and this protection was associated with SIV Env-specific antibody responses (12). The regulation of Ad vector-elicited antibody responses, however, remains poorly understood.

The induction of antibody responses is typically dependent on CD4⁺ T cell help (13). In the context of antibody induction, CD4⁺ T cells primary function to promote and maintain B cell germinal center responses. A critical mediator of CD4⁺ T cell help to B cells is via the engagement of the CD40 signaling pathway (14). Studies using model antigens have demonstrated that the proper development of germinal center responses is a dynamic process where CD4⁺ T cell help, via CD40, is provided for several days (15, 16). We have recently described that following Ad vector immunization, CD4⁺ T cell help is required for a month post-immunization to properly induce CD8⁺ T cell responses (17). Thus, we sought to determine whether prolonged CD4⁺ T cell help is also required following Ad vector immunization for the induction of transgene-specific antibody responses.

In this study, we identified that following Ad vector immunization, CD4⁺ T cell help is required for ten days post-immunization to induce optimal antigen-specific antibody titers.

Unexpectedly, we also observed that CD4⁺ T cell depletion prior to immunization did not result in a permanent ablation of antigen-specific antibody responses. Instead, the induction of antibody responses was simply delayed until the time at which the CD4⁺ T cells began to recover. These delayed antibody responses exhibited no apparent functional defects. Delayed antigen-specific antibody responses were also observed using a protein immunogen formulated in an alum-based adjuvant. Thus, in contrast to the current model that the depletion of CD4⁺ T cells at the time of antigen exposure results in tolerance (18-22), we demonstrate that functional antibody responses can be induced at a time separate from immunization with Ad vector or protein-based vaccines in the context of transient CD4⁺ T cell depletion.

MATERIALS AND METHODS

Mice, vectors, proteins, and viruses. Six- to ten-week-old C57BL/6, B6.129S2-H2^{dlAb1-Ea}/J (MHC II KO), B6.129S2-Cd40lg^{tm1Imx}/J (CD40L KO), and B6.129P2-Cd40^{tm1Kik}/J (CD40 KO) mice were purchased from (Jackson Laboratory, Bar Harbor, ME). Thymectomized C57BL/6 underwent adult thymectomy at the Jackson Laboratory (Bar Harbor, ME). Mice were immunized intramuscularly (i.m.) in a volume of 100 μ l divided between the two quadriceps. The previously described E1/E3 deleted Ad26-SIV_{mac239} Env or Ad5-SIV_{mac239} Env were used at a dose of 10⁹ or 10¹⁰ vp (23). SIV Env 32H gp140 was used at 50 μ g + 100 μ g Adju-Phos (Brenntag) (24). All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Beth Israel Deaconess Medical Center.

Monoclonal antibodies. The monoclonal α CD4 antibody (GK1.5; BioXcell) was administered by two intraperitoneal injections of 500 μ g on consecutive days. To maintain depletion of CD4⁺ T cells, where applicable, GK1.5 was readministered in two 500 μ g doses every 14 to 21 days.

Serum collection and tissue processing. Blood was collected and serum was clarified by collection of the supernatant following centrifugation at 10000 rpm for 5 minutes. Two centrifugation steps were performed and following clarification serum was stored at -20 °C for further analysis. Splenic and iliac lymph node mononuclear cells were harvested as previously described (25). Tissues were ground through a 70 μ m strainer (Fisher Scientific), red blood cells were lysed by Ammonium-Chloride-Potassium (ACK) treatment for three minutes, and debris was removed by subsequent filtering through a 30 μ m filter (Miltenyi Biotec).

Endpoint ELISA. Endpoint enzyme-link immunosorbent assays (ELISAs) were performed as previously described (26). Briefly, ELISA plates were coated overnight with 1 μ g/ml of SIV_{mac239} gp140 Env. Plates were blocked for 4 hrs with PBS + 2% BSA + 0.05% Tween 20. Mouse sera

was serially diluted, added to the ELISA plate, and incubated for 1 hr. Bound sera was detected by 1 hr incubation with peroxidase-conjugated, affinity-purified rabbit anti-mouse secondary antibody diluted 1:2000 (Jackson ImmunoResearch Laboratories). Plates were developed and read on a Spectramax Plus ELISA plate reader using Softmax Pro 4.7.1 software (Molecular Devices). Positive titers were defined as the greatest serum dilution with OD >2-fold above naïve negative control serum OD.

Isotype and urea disruption ELISAs. Isotype and urea disruption ELISAs were performed as previously described (27, 28). A semi-quantitative immunoglobulin ELISA protocol described previously was followed. Briefly, 0.5 µg/mL SIV_{mac239} gp140 Env coated ELISA plates were blocked with 1% Bovine Serum Albumin (BSA)/0.05% Tween in PBS (PBS-T). After washing, diluted samples were added to the plates for 1 hr before washing and the addition of a 1:4000 dilution of either anti-mouse IgG conjugated to Horseradish peroxidase (HRP), IgG1-HRP, or IgG2a-HRP (Southern Biotech). Standards consisted of coating ELISA plate wells with anti-mouse Kappa (1:3200) and Lambda (1:3200) light chain (Serotec, UK), blocking, washing, and then adding a standard five-fold dilution series of purified IgG or IgA (Southern Biotech, UK) starting at 1000 ng/mL. Samples and standards were developed using 3,3',5,5'-Tetramethylbenzidine (TMB) and the reaction was stopped after 5 min with Stop solution (Insight Biotechnologies, UK). Absorbance was read on a Spectramax Plus ELISA plate reader (Molecular Devices) with SoftMax Pro 4.7.1 software.

The avidity indices of serum samples were determined by their antibody-antigen binding resistance to 8 M urea. Serum samples were pre-diluted to give an OD_{450 nm} readout between 1.0 and 1.5 in an ELISA and were added to SIV_{mac239} gp140 Env coated plates. Plates were then washed three times with either PBS-T or 8 M urea in PBS-T, before incubating with anti-mouse

IgG-HRP. Samples were developed with TMB as described above. The avidity index was calculated as the percentage of urea treated OD_{450 nm}/ PBS-T OD_{450 nm}.

SIV Neutralization assay. SIV-specific neutralization assays were performed as previously described using the TZM.bl neutralization assay (29). Briefly, mouse sera was serially diluted and incubated for one hour with the Tier 1A neutralization sensitive pseudotype virus SIVmac251.TCLA.15 and was subsequently added to TZM.bl cells. A pseudotyped virus expressing the envelope gene of murine leukemia virus (MuLV) was used as a negative control. After 48 hrs cells were lysed and relative luminescence units were quantified. ID₅₀ was calculated as the serum concentration that reduced relative luminescence units by 50% relative to a no sera control well. Pseudotyped viruses were prepared as previously described (30).

Germinal Center B cells. Single cell suspensions of iliac LN mononuclear cells were blocked with TruStain fcX (anti-mouse CD16/CD32) antibodies (Biolegend) for 10 min at 4 °C. Cells were washed and stained for 30 min at 4 °C with α CD3 ϵ (145-2C11), α CD19 (6D5), α Fas (15A7), peanut agglutinin (PNA; Vector Labs), α IgM (RMM-1), and α IgD (11-26c.2a). Dead cells were excluded by the use of a vital exclusion dye (Life Technologies). All antibodies were purchased from BD Biosciences, Affymetrix, or BioLegend, unless noted otherwise. Germinal center B cells were identified by flow cytometry as Fas⁺ PNA⁺ CD19⁺, as previously described (31). Samples were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v9.8.3 (Treestar).

Statistics. Mann-Whitney U tests were performed with a significance cutoff of P<0.05.

RESULTS

Ad vector-elicited antibody responses are delayed following the depletion of CD4⁺ T cells at immunization.

We first sought to determine when CD4⁺ T cell help is required for the induction of transgene antigen-specific antibody responses by Ad vector vaccination. To accomplish this, C57BL/6 mice were immunized i.m. with 10¹⁰ vp of Ad26-SIV Env and depleted of CD4⁺ T cells by the administration of the α CD4 antibody GK1.5 on days -1, 3, 7, 10, 14, 21, or 28 post-immunization or left untreated, as a control (Fig. 4.1A). In untreated control mice, robust SIV Env-specific serum antibody titers were detected on day 14 post-immunization and were maintained for at least 90 days (Fig. 4.1B). Depletion of CD4⁺ T cells prior to immunization resulted in nearly undetectable Env-specific antibody responses at day 14 and 30 post-immunization (Fig. 4.1B). Depletion of CD4⁺ T cells between day 3 and 10 post-immunization had progressively less of an impact on Env-specific antibody titers as measured on day 30, and depletion of CD4⁺ T cells on or after day 14 post-immunization had no impact on antibody titers as measured on day 30 (Fig. 4.1B). As expected, the decrease in antibody titers observed following depletion of CD4⁺ T cells was associated with reductions in the frequency of germinal center B cells in the iliac (draining) lymph nodes (LNs) on day 14 post-immunization (Fig. 4.1C and 4.1D). Signaling by CD4⁺ T cells through CD40 is a well-described mechanism by which CD4⁺ T cells promote antibody responses (16). We confirmed that CD40 was an important signaling pathway for antigen-specific antibody responses following Ad vector immunization, as absence of CD40L or CD40 resulted in a complete abolishment of Env-specific antibody responses on day 30 post-immunization (Fig. 4.1E). Thus, following Ad vector immunization, induction of antigen-

specific antibody responses requires CD4⁺ T cell help to promote germinal center responses via CD40-derived signals, consistent with other experimental systems (32).

We next sought to determine whether CD4⁺ T cells regulated the maintenance of Ad vector-induced antibody responses. Unexpectedly, when Env-specific antibody responses were measured on day 60 and 90 post-immunization, we observed no significant difference in antibody titers between mice that had been previously depleted of CD4⁺ T cells and the untreated control animals (Fig. 4.1B, bottom panels). Despite the lack of detectable serum antibody responses on day 30 post-immunization in mice depleted of CD4⁺ T cells prior to immunization, these same animals developed Env-specific antibody titers of equivalent magnitude to untreated control mice by day 60 post-immunization. We define the antibody responses that develop on day 60 post-immunization after depletion of CD4⁺ T cells as “delayed antibody responses”. Similar delayed Env-specific antibody responses were also observed in mice depleted of CD4⁺ T cells between day 3 and 10 post-immunization. Thus, depletion of CD4⁺ T cells immediately prior to, or after, immunization resulted in a delay but did not prevent the induction of Env-specific antibody responses.

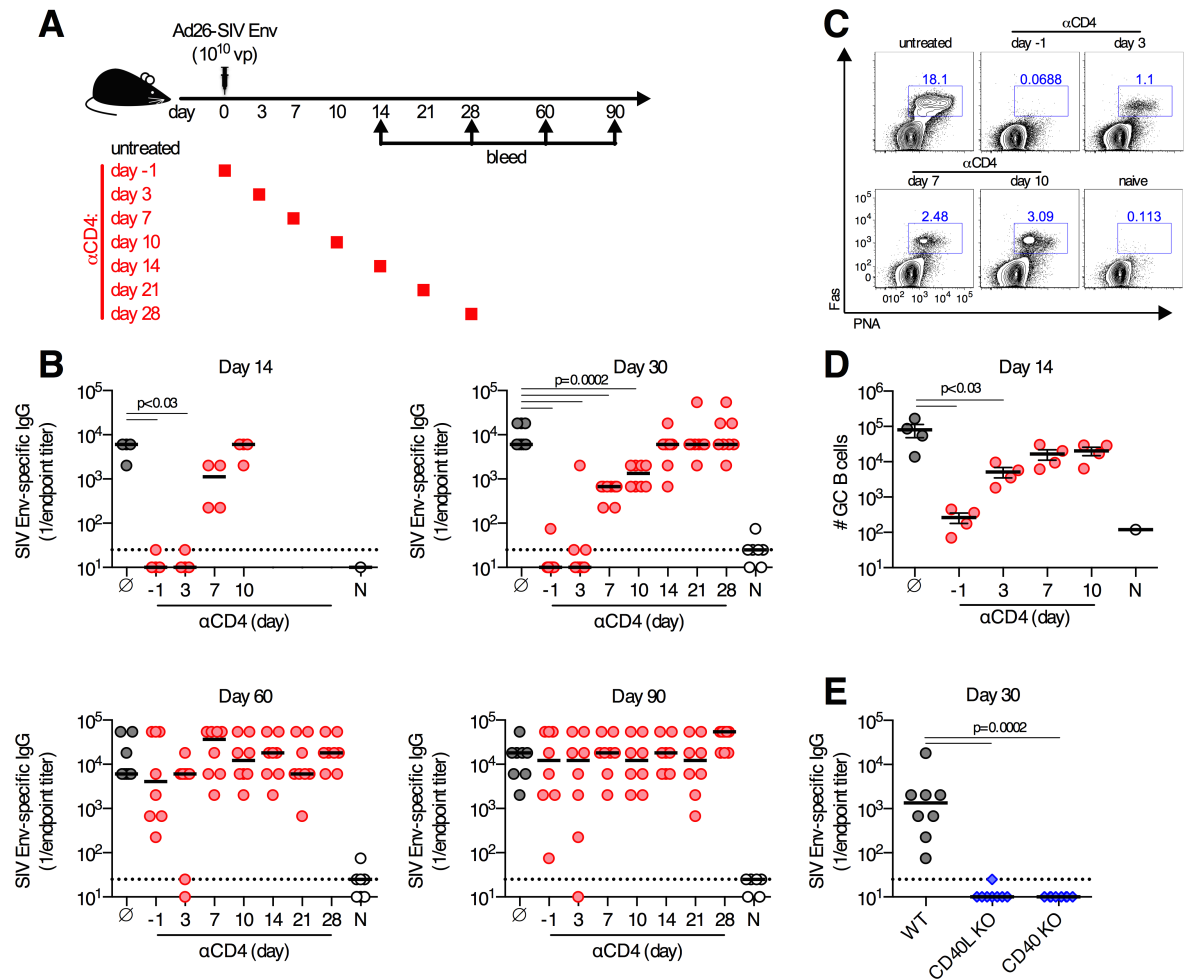


Figure 4.1. Delayed development of antibody responses following depletion of CD4⁺ T cells. (A) C57BL/6 mice were depleted of CD4⁺ T cells at the indicated day or left untreated and immunized intramuscularly with 10^{10} vp of Ad26-SIV Env. (B) SIV Env-specific serum binding antibody titers at the indicated day post-immunization. Grey, no α CD4 treatment; Red, α CD4 given on the indicated day; white, naive animals. (C and D) Representative flow cytometry plots (C) and absolute number (D) of germinal center B cell responses in the iliac LN on day 14 post-immunization. Gated on CD3 ϵ ⁺CD19⁺ cells. (E) SIV Env-specific serum binding antibody titers in WT, CD40L KO, or CD40 KO mice immunized with 10^9 vp of Ad26-SIV Env. Each dot represents an individual mouse and the line is median (B and E) or mean \pm SEM (D).

Delayed antibody responses following CD4⁺ T cell depletion are functional.

We sought to determine whether the delayed antibody responses observed after day 60 in mice depleted of CD4⁺ T cells prior to immunization are functionally normal. We first assessed if the delayed antibody responses exhibited alterations in their isotype proportion. The concentration of IgG2a isotype Env-specific antibodies increased from day 30 to 90 post-immunization in α CD4 treated mice, and by day 90 only a statistical trend for differences in antibody concentration in α CD4 treated versus untreated mice were observed ($p=0.06$; Fig. 4.2A). Similarly, IgG1 isotype Env-specific antibody concentration increased in α CD4 treated mice between day 30 and 90, and reached equivalent antibody concentrations as in untreated control mice by day 90 (untreated median = 1409 ng/ml and α CD4 median = 975.5 ng/ml; $p=0.6$; Fig. 4.2B). On both day 60 and 90 post-immunization, the ratio of IgG2a to IgG1 isotype antibodies was equivalent between α CD4 treated and untreated groups ($p=0.3$; Fig. 4.2C). Finally, we sought to determine if the delayed antibody response that developed following depletion of CD4⁺ T cells would have reduced antigen-binding avidity as measured using a urea disruption assay (27). Env-specific antibodies from day 60 or 90 post-immunization of α CD4 treated and untreated mice displayed no significant differences in Env-binding avidity by urea disruption ELISA (Fig. 4.2D). Thus, the delayed antibody responses exhibited no significant abnormalities in their isotype or binding avidity.

To further investigate the functionality of these delayed antibody responses we assessed whether these responses could expand after a boosting immunization. C57BL/6 mice were immunized i.m. with 10^{10} vp of Ad26-SIV Env and depleted of CD4⁺ T cells on day -1 or left untreated as a control (Fig. 4.3A). Four months after the primary immunization, mice were boosted i.m. with 10^{10} vp of Ad5-SIV Env (Fig. 4.3A). One month after the boosting

immunization the α CD4 antibody treated and untreated mice had equivalent median SIV Env-specific endpoint titers (Fig. 4.3B), which reflected equivalent mean fold-expansion of 54-fold and 43-fold, respectively ($p=0.8$; Fig. 4.3C). As a final measure of functional capacity, we assessed the ability of these delayed antibodies to neutralize an SIV Env-expressing pseudovirus. No SIV-specific neutralizing antibodies in α CD4 treated mice were detected at one month post-immunization (Fig. 4.3D), which is consistent with the lack of Env-specific binding antibodies at this time point (Fig. 4.1B). However, by four months post-immunization, α CD4 treated mice had median neutralizing antibody titers that were not significantly different from those in undepleted vaccinated mice ($p=0.9$; Fig. 4.3D). In both groups, neutralizing antibody titers increased following the boosting immunization, and again no differences between the two groups were observed (Fig. 4.3D). Collectively, these data demonstrate that the delayed antibody responses that developed following depletion of CD4⁺ T cells at the time of primary Ad vector immunization have no detectable defects in boosting capacity, ability to acquire functional neutralization capacity, isotype proportion, or antigen-binding avidity.

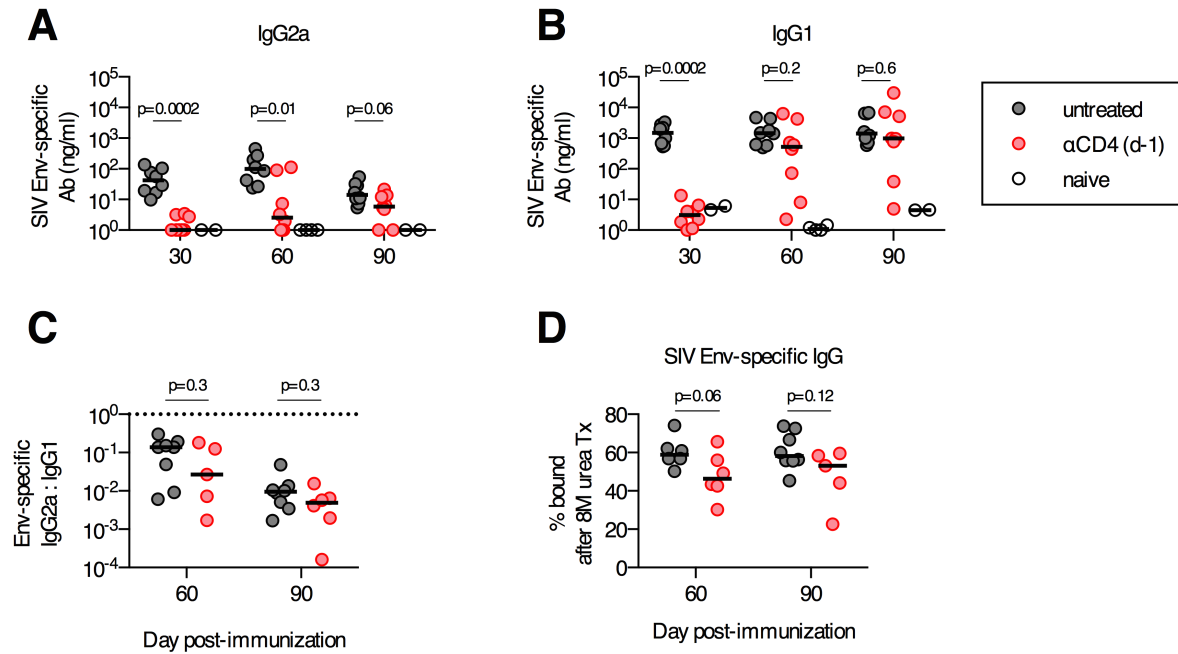


Figure 4.2. Transient depletion of CD4⁺ T cells at priming does not alter the isotype distribution or the antigen-binding avidity of the delayed antibody responses. C57BL/6 mice were depleted of CD4⁺ T cells or left untreated and immunized intramuscularly with 10^{10} vp of Ad26-SIV Env. Concentration of serum SIV Env-specific IgG2a (**A**) or IgG1 (**B**). (**C**) Ratio of SIV Env-specific IgG2a to IgG1 serum antibody titers. (**D**) Avidity of SIV Env-specific serum antibodies as determined by urea disruption assay. Each dot represents an individual mouse and for (**A-C**) median or (**D**) mean \pm SEM is indicated by the line.

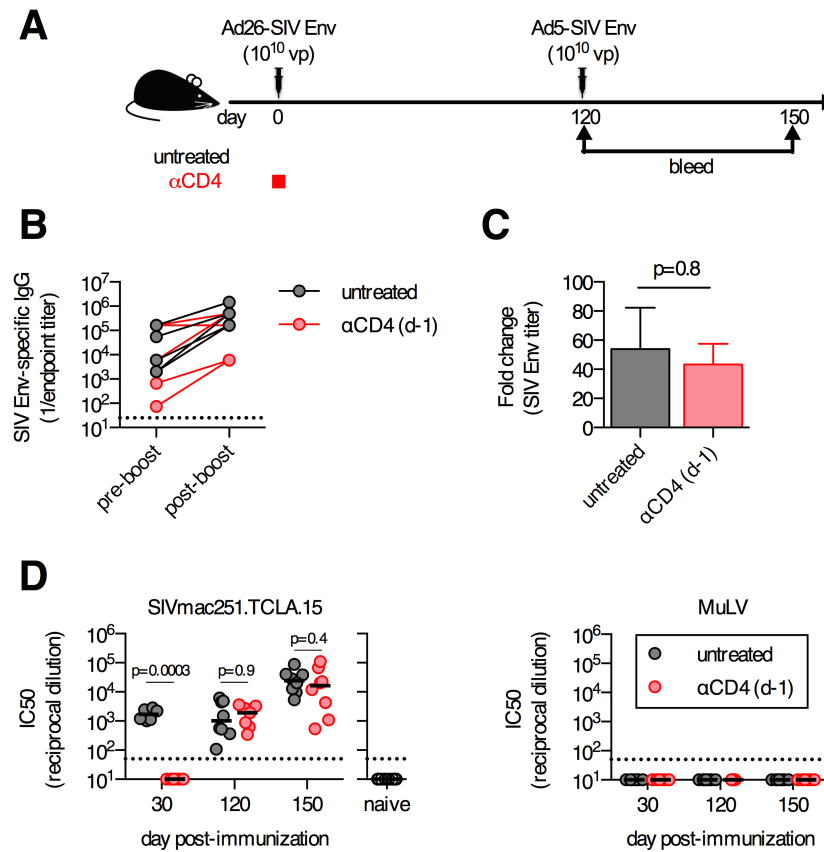


Figure 4.3. Boosting capacity and functional neutralization capacity of the delayed antibody responses that develop following transient CD4⁺ T cell depletion. (A) C57BL/6 mice were depleted of CD4⁺ T cells or left untreated and immunized intramuscularly with 10^{10} vp of Ad26-SIV Env and boosted four months post-prime with 10^{10} vp of Ad5-SIV Env. (B) SIV Env-specific antibody titers prior to and following boosting immunization. (C) Fold change in SIV Env-specific antibody responses pre- to post-boost. (D) Serum neutralization capacity of Tier 1A SIVmac251.TCLA.15 Env-expressing pseudoviruses or a MuLV negative control pseudovirus. Each dot represents an individual mouse and for (C) mean \pm SEM or (D) median is indicated by a line.

Continuous absence of CD4⁺ T cells prevents the development of antibody responses.

We hypothesized that the development of delayed antibody responses following CD4⁺ T cell depletion reflected the transient nature of CD4⁺ T cell depletion following a single regimen of α CD4 antibody treatment. To test this, C57BL/6 mice were immunized i.m. with 10^{10} vp of Ad26-SIV Env and divided into three experimental groups: 1) treated with α CD4 antibody on day -1 (α CD4 at prime); 2) treated with α CD4 antibody on day -1 and again every 14 to 21 days (α CD4 repeated); 3) untreated controls (Fig. 4.4A). Administration of α CD4 antibody at prime resulted in complete depletion of CD4⁺ T cells for at least 30 days, but the CD4⁺ T cell population had largely recovered by day 60 (Fig. 4.4B). In contrast, repeated administration of α CD4 antibody maintained depletion of CD4⁺ T cells for at least 60 days (Fig. 4.4B). Repeated administration of α CD4 antibody prevented the development of SIV Env-specific antibody responses in 18 of 20 mice by day 60 post-immunization ($p < 0.001$; Fig. 4.4C). Consistent with the observation that repeated administration of α CD4 antibody prevented the development of a delayed antibody response, MHC II KO mice, which permanently lack CD4⁺ T cells, had no SIV Env-specific antibody responses on day 60 post-immunization (Fig. 4.4D). Thus, the recovery of CD4⁺ T cells following α CD4 antibody treatment is necessary for the development of these delayed antibody responses.

We next tested the possibility that the development of delayed antibody responses is due to incomplete depletion of CD4⁺ T cells following treatment, thus resulting in signals from residual primed antigen-specific CD4⁺ T cells. To test this, adult C57BL/6 mice were thymectomized and depleted of CD4⁺ T cells prior to immunization with Ad26-SIV Env. A single administration of the α CD4 antibody to thymectomized mice results in a permanent depletion of CD4⁺ T cells, due to an inability to generate new CD4⁺ T cells (data not shown). On

day 60 post-immunization, no SIV Env-specific antibodies were detected in thymectomized mice treated at priming with α CD4 antibody (Fig. 4.4E). Thus, our data show that any residual CD4⁺ T cells are insufficient for the generation of a delayed antibody response. Moreover, our data further highlight that *de novo* generation of CD4⁺ T cells by the thymus is required for the delayed antibody response to develop. Collectively, these data demonstrate that the delayed antibody response that develops following α CD4 antibody treatment requires thymus-driven reconstitution of the CD4⁺ T cell compartment.

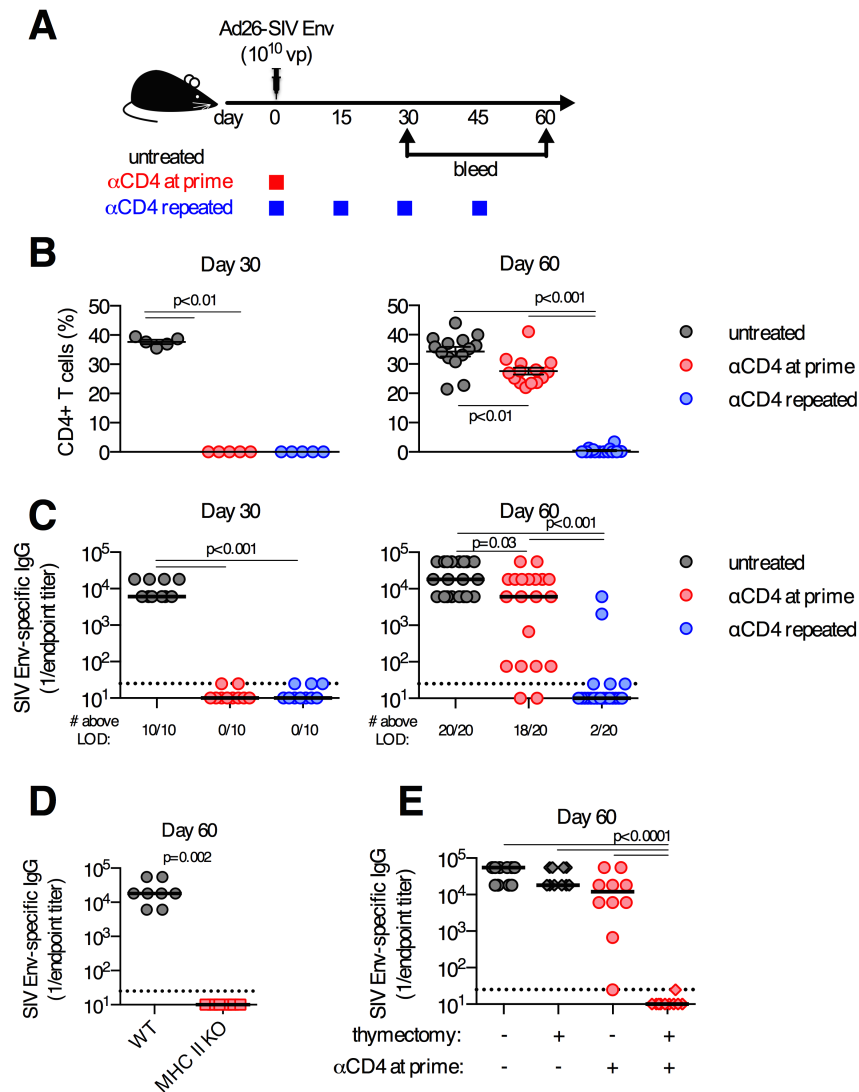


Figure 4.4. Repeated depletion of CD4⁺ T cells prevents the development of the delayed antibody response. (A) C57BL/6 mice were depleted of CD4⁺ T cells a single time (α CD4 at prime, red circles), depleted of CD4⁺ T cells repeatedly (α CD4 repeated, blue circles) or left untreated (grey circles) and immunized intramuscularly with 10^{10} vp of Ad26-SIV Env. (B) Frequency of CD4 T cells in the iliac (draining) LNs. (C) SIV Env-specific serum binding antibody titers. (D) Serum SIV Env-specific antibody titers on day 60 post-immunization from WT or MHC II KO mice immunized with 10^{10} vp of Ad26-SIV Env. (E) Serum SIV Env-specific antibody titers on day 60 post-immunization from mice having undergone adult thymectomy, or not, and treated with α CD4 antibody, or not, as indicated. Each dot represents an individual mouse and for (B) mean \pm SEM or (C-E) median is indicated by a line.

CD4⁺ T cell rebound following transient depletion induces normal germinal center responses.

As CD4⁺ T cells were critical for promoting the formation of germinal center responses following Ad vector immunization (Fig. 4.1), we hypothesized that when CD4⁺ T cells recovered following transient depletion, *de novo* germinal center responses would be induced. On day 30 post-immunization no germinal center B cells were detected in the iliac lymph nodes of Ad26-SIV Env immunized mice treated with α CD4 antibody at priming (Fig. 4.5A). However, by day 60 post-immunization, when the frequency of CD4⁺ T cells had returned to near basal levels in these animals, robust germinal center responses were observed. These responses were equivalent in frequency and absolute number of cells to the germinal center responses measured in untreated control mice (Fig. 4.5A-C). These germinal center B cells also displayed the normal down-regulation of IgM and IgD (Fig. 4.5D). Recovery of CD4⁺ T cells was required for the induction of these delayed germinal center responses as sustained depletion of CD4⁺ T cells prevented germinal centers from developing (Fig. 4.5A-C). Thus, recovery of CD4⁺ T cells results in the generation and expansion of germinal centers, and the development of the delayed antibody response.

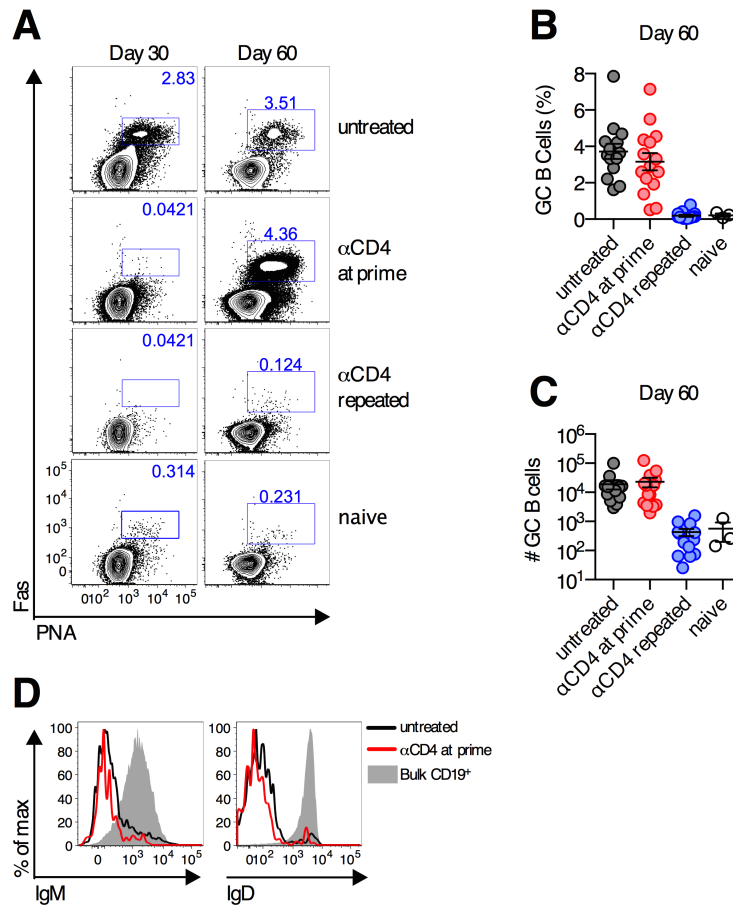


Figure 4.5. Germinal center B cell responses develop following transient depletion of CD4⁺ T cells. C57BL/6 mice were depleted of CD4 T cells a single time (α CD4 at prime, red circles), depleted of CD4 T cells repeatedly (α CD4 repeated, blue circles) or left untreated (grey circles) and immunized intramuscularly with 10^{10} vp of Ad26-SIV Env. **(A-C)** Representative flow cytometry plots **(A)**, group average percent **(B)** or absolute number **(C)** of germinal center B cells in the iliac (draining) LNs on day 60 post-immunization. **(D)** Expression of IgM and IgD on germinal center B cells on day 60-post-immunization. Each dot represents an individual mouse and mean \pm SEM is indicated by a line.

Development of delayed antibody responses occurs following soluble protein immunization.

We sought to identify other immunization regimens where delayed antibody responses may occur following transient depletion of CD4⁺ T cells. Therefore, we tested adjuvanted soluble protein immunogens as an alternate vaccine platform. C57BL/6 mice were immunized i.m. with 50 µg of trimeric SIV Env gp140 protein formulated in 100 µg of Adju-Phos adjuvant and mice were either left untreated, depleted of CD4⁺ T cells at priming, or repeatedly depleted of CD4⁺ T cells (Fig. 4.6). This immunization regimen induced robust Env-specific titers at day 30 post-immunization in untreated control mice (Fig. 4.6), and titers were maintained to day 60. In mice transiently depleted of CD4⁺ T cells, no Env-specific antibody responses were detected on day 30 post-immunization, but by 60 post-immunization these animals had equivalent titers to the untreated control animals (Fig. 4.6). Continuous depletion of CD4⁺ T cells by repeated administration of αCD4 antibody prevented the development of Env-specific antibodies on day 60 (Fig. 4.6). These data demonstrate that a delayed antibody response can occur in the context of multiple immunization regimens and is not a unique characteristic of immunization with replication-incompetent Ad vectors.

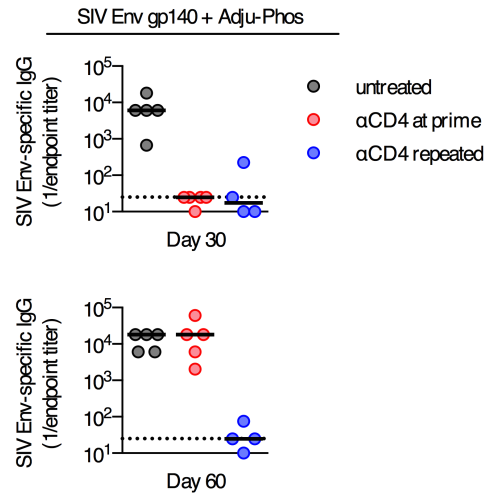


Figure 4.6. Delayed antibody responses develop following immunization with an adjuvanted soluble protein formulated in Adju-Phos but not following immunization with a replication-incompetent poxvirus vector. SIV Env-specific serum antibody titers from C57BL/6 mice immunized intramuscularly with 50 μ g of SIV Env gp140 + Adju-Phos and depleted of CD4⁺ T cells at the time of immunization (α CD4 at prime), or continuously depleted of CD4⁺ T cells (α CD4 repeated), or left untreated. Each dot represents an individual mouse and the line is median.

DISCUSSION

In this study we demonstrate that the induction of transgene antigen-specific antibody responses by vaccination can be temporally separated from the time of immunization by the transient depletion of CD4⁺ T cells. CD4⁺ T cell help was required for the induction of antibody responses. However, the transient absence of CD4⁺ T cells at the time of Ad vector immunization only delayed the development of an antibody response until the time at which the CD4⁺ T cell population returned, and we identified no functional defects in these delayed antibody responses once they had developed. This is unexpected because previous studies have suggested that absence of CD4⁺ T cells at the time of antigen exposure would induce tolerance (18-22). These findings have implications for understating the biology of Ad vector vaccines.

CD4⁺ T cells have many well-established roles in regulating antibody responses against an array of antigens (13). One key aspect of this process is sustained provision of positive signals to responding B cells by CD4⁺ T cells (16). Consistent with these findings in other systems, the induction of robust antibody responses by Ad vector immunization requires sustained CD4⁺ T cell help for ten days following immunization (Fig. 4.1). Depletion of CD4⁺ T cells at the time of antigen administration was initially suggested as a means to induce B cell tolerance, since antigen-specific antibody responses did not develop even after the CD4⁺ T cell population recovered (18-22), and continues to be investigated for this purpose (33). In contrast to these reports, we demonstrate that transient CD4⁺ T cell depletion at the time of immunization with an Ad vector or a protein formulated with alum did not induce tolerance to the vaccine antigen. Instead, once the CD4⁺ T cell compartment is reconstituted, robust antigen-specific antibody responses develop without the need to experimentally re-administer antigen, and these responses appear functionally indistinguishable by our measures from the response induced if CD4⁺ T cells

are present at the time of immunization. Thus, while CD4⁺ T cell help is required for the induction of antigen-specific antibody responses elicited by Ad vectors and soluble proteins formulated in an alum-based adjuvant, this help need not be provided at the time of immunization.

We hypothesize that tolerance does not occur following transient CD4⁺ T cell depletion in the context of Ad vector immunization because antigen-specific CD4⁺ T cell responses may develop when the CD4⁺ T cell population recovers. Transient depletion of CD4⁺ T cells at the time of infection with LCMV Cl-13, a life-long chronic viral infection (34), has also been shown to result in a permanent absence of LCMV-specific antibody responses (22) and (data not shown). In the context of LCMV Cl-13 infection, when CD4⁺ T cells are transiently depleted, the recovering population is not LCMV-specific (34, 35), possibly due to the presence of viral antigen in the thymus, which may result in induction of central tolerance (36). However, adoptive transfer of naive LCMV-specific, but not OVA-specific, CD4⁺ T cells into LCMV Cl-13-infected mice results in the generation of the LCMV-specific antibody responses (22). In the context of chronic LCMV infection, the absence of a delayed antibody response appears to be due to a lack of antigen-specific CD4⁺ T cells upon recovery of CD4⁺ T cell population. These data suggest that generation of antigen-specific CD4⁺ T cells is necessary for the development of a delayed antibody response, and thereby prevent tolerance. Future studies will interrogate this proposed model in the context of Ad vector immunization.

How B cell priming occurs with regards to a delayed antibody response remains unclear. Even in response to T cell-dependent antigens, the initial activation of naive B cells by cognate binding of antigen to the B cell receptor (BCR) occurs prior to interaction with T cells (37-39). Thus, it is possible that the delayed antibody response that occurs following transient depletion

of CD4⁺ T cells is the result of these antigen-experienced B cells receiving the necessary activation signals following reconstitution of the CD4⁺ T cell compartment. Alternatively, the delayed antibody response may instead involve B cells that recognize antigen and become activated at a time more contemporaneous with the recovery of CD4⁺ T cells. Future experiments will be required to distinguish between these two possibilities and will enhance our understanding of this phenomenon.

The strong dependence of robust antibody responses on CD4⁺ T cell help has important clinical implications. Vaccination in the setting of reduced CD4⁺ T cell levels, such as in AIDS patients or following hematopoietic stem cell transplantation, is challenging as many licensed vaccines show a decreased ability to induce antibody responses in these populations (40-44). In this study, we demonstrate that following immunization with specific candidate vaccine platforms, it is possible to develop high titer, functional antibody responses even when CD4⁺ T cells are absent at the time of immunization. Thus, Ad vectors may be particularly appealing vaccine platforms to give in the setting of low CD4⁺ T cell counts where highly active anti-retroviral therapy or immune reconstitution can be utilized to reconstitute CD4⁺ T cell levels post-vaccination, as vector-elicited antibody responses can be rescued at least one month post-vaccination.

In conclusion, we have shown the role of CD4⁺ T cell responses in helping to elicit serum antigen-specific antibody responses following immunization with non-replicating Ad vectors and adjuvanted soluble protein immunogens. Our data demonstrate that antibody responses can be induced at a time separated from immunization by manipulation of CD4⁺ T cells. These findings demonstrate that provision of critical CD4⁺ T cell-derived help signals to B cells can occur substantially after the time of initial antigen exposure. Future studies will have to determine if

manipulation of this process may be a way to regulate the exact timing of antibody induction following vaccination.

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Chapter 5: Conclusions

TIMING OF CD4⁺ T CELL HELP IS CRITICAL

The unifying theme of this thesis is that the timing of CD4⁺ T cell help to CD8⁺ T cells and B cells is critical for the generation of optimal, fully functional immune responses. The data presented in chapters 2 and 3 allow for a very clear picture of the exact timing of CD4⁺ T cell help for the generation of CD8⁺ T cell responses following Ad vector immunization. Furthermore, the data presented in chapter 3 provide mechanistic insights into how CD4⁺ T cells regulate Ad vector-elicited CD8⁺ T cell responses. Finally, the data in chapter 4 provide details on the timing of CD4⁺ T cell help to B cells to promote the generation of antibody responses, and demonstrate that CD4⁺ T cell help can be temporally separated from the time of immunization.

Timing of CD4⁺ T cell help to CD8⁺ T cells

Following immunization or natural infection with a subset of agents, CD4⁺ T cell help is critical for the optimal expansion of primary CD8⁺ T cell responses. This class of pathogens/immunogens includes vaccinia virus infection, and immunization with antigen-loaded splenocytes, DNA plasmids, and Adenovirus serotype 5 (Ad5) vectors (1-4). In contrast, some pathogens, such as lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes*, can induce robust primary CD8⁺ T cell responses in the absence of CD4⁺ T cells (5-7). I, and others (8), postulate that the difference between these two classes of pathogens/immunizations with regards to degree of CD4⁺ T cell independence is due to differences in innate immune stimulatory capacity. Namely, pathogens that induce robust innate immune responses do not require CD4⁺ T cell help for primary CD8⁺ T cell responses, while those that induce modest innate immune activation do. In support of this model, administration of poly(I:C), to induce type I interferon production, can rescue the proliferation of unhelped CD8⁺ T cells following

vaccinia virus infection (9). For both vaccinia virus infection and antigen-loaded splenocyte immunization, a primary mechanism of CD4⁺ T cell help is maturation of dendritic cells via the CD40 signaling pathway (1, 10). However, a mechanism of CD4⁺ T cell help in the context of Ad5 vector immunization has not been defined.

The applicability of the CD40-dependent model of CD4⁺ T cell help to Ad vector-elicited immune responses was investigated. Adenovirus serotype 26 (Ad26)-induced CD8⁺ T cell responses, like Ad5, require CD4⁺ T cell help at priming. However, no role was identified for CD40 signaling in modulating expansion of primary CD8⁺ T cell responses following Ad26 (Fig. 2.8) or Ad5 (data not shown) vector immunization. No prior studies have identified the timing of CD4⁺ T cell help in the context of the primary CD8⁺ T cell responses. In prior studies, CD4 knockout (KO) or MHC class II KO mice, which permanently lack CD4⁺ T cells, were used, or CD4⁺ T cells were depleted by monoclonal α CD4 antibody administration prior to immunization. Thus, I postulated that identifying the timing of CD4⁺ T cell help for promoting primary CD8⁺ T cell responses by Ad vector immunization would provide insights into how CD4⁺ T cell help is mediated.

Fine mapping of the timing of CD4⁺ T cell help was performed using α CD4 antibody-mediated depletion of CD4⁺ T cells, and reconstitution of CD4⁺ T cells by adoptive transfer of antigen-specific CD4⁺ T cells. Post-Ad26 immunization, CD4⁺ T cell help is required for eight days after immunization to drive maximal expansion of the primary CD8⁺ T cell response. The impact depletion of CD4⁺ T cells has on the magnitude of the CD8⁺ T cell response wanes smoothly from absolute ablation when cells are depleted prior to immunization to no impact when cells are depleted on day nine post-immunization. To complete the fine mapping of the timing of CD4⁺ T cell help, the reciprocal experiment where CD4⁺ T cells were reconstituted at

various times post-immunization was performed. Following Ad5 vector immunization, adoptive transfer of antigen-specific CD4⁺ T cells to mice lacking CD4⁺ T cells partially rescues proliferation of CD8⁺ T cells if the transfer is performed on day 3, but not on day 7 post-immunization. Thus, provision of CD4⁺ T cell help beginning a few days after initial antigen-exposure can partially rescue defects in CD8⁺ T cell proliferation. In conclusion, CD4⁺ T cell help is required for a prolonged period following immunization but is not strictly necessary at the time of immunization, and that help is being continuously provided during this period.

There are a few minor technical points to consider when interpreting these results. In these experiments, naïve antigen-specific CD4⁺ T cells were transferred, so the partial rescue of CD8⁺ T cell responses may reflect a lag in provision of CD4⁺ T cell help due to the need for the CD4⁺ T cells to become activated. Alternatively, it may suggest that multiple CD4⁺ T cell-derived signals are required for optimal CD8⁺ T cell expansion, and that the first of these signals is provided prior to day three post-immunization. A limitation of comparing these data to those generated in the α CD4 antibody-mediated depletion experiments is that the two immunizing vector serotype are different, and the CD8⁺ T cell responses induced by these vectors expand with different kinetics (11, 12). Thus, the exact time when CD4⁺ T cell help is no longer needed may differ slightly between the two experimental systems. Regardless of these minor technical points, these data provide the greatest temporal detail on the timing of CD4⁺ T cell help to CD8⁺ T cells in any system.

These fine mapping data of the timing of CD4⁺ T cell help provides insights into potential mechanisms of CD4⁺ T cell help. The lack of a sharp demarcation between one day and the next with regards to the impact of CD4⁺ T cell depletion on the magnitude of the CD8⁺ T cell response strongly argues against a mechanism of CD4⁺ T cell help that involves programming of

CD8⁺ T cells to subsequently undergo autonomous CD4⁺ T cell-independent proliferation. Such a process of cell autonomous proliferation has been shown to occur following CD8⁺ T cell recognition of cognate antigen (13-15). Instead, the prolonged, gradually declining need for CD4⁺ T cell help suggests a continuous provision of CD4⁺ T cell-derived helper signals. Furthermore, given the relative overabundance of antigen-specific CD8⁺ T cells compared to antigen-specific CD4⁺ T cells following Ad vector immunization (16), it seems unlikely that direct cell-cell contact between CD4⁺ T cells and CD8⁺ T cells could be a major mechanism of CD4⁺ T cell help. Consistent with this hypothesis, the absence of CD4⁺ T cells results in strikingly decreased expression of the high-affinity IL-2R α subunit (CD25) on CD8⁺ T cells. Importantly, specific absence of CD25 expression on CD8⁺ T cells results in profound impairment in expansion of these cells post-Ad vector immunization, and administration of recombinant IL-2 (rIL-2) can partially rescue the proliferative defects of unhelped CD8⁺ T cells. These data suggest that CD4⁺ T cell-derived IL-2 is a critical factor in driving optimal expansion of CD8⁺ T cell responses following Ad vector immunization. Further experimentation to specifically abolish IL-2 production by CD4⁺ T cells will be required to validate this model.

Pro-inflammatory “signal 3” cytokines are usually required for the induction of optimal CD8⁺ T cell responses, and these cytokines include IL-12, type I interferons, and IL-21 (17-20). However, following Ad vector immunization, neither absence of type I interferons nor the absence of IL-21 substantially alters CD8⁺ T cell primary expansion (21, 22). Additionally, no IL-12 is detected in serum following Ad vector immunization of non-human primates (23), which suggests that IL-12 does not play a major role in regulating Ad vector-elicited CD8⁺ T cell responses. In summary, it does not appear that any of the described “signal 3” cytokines regulate

Ad vector-elicited CD8⁺ T cell proliferation. Instead, IL-2, likely produced by CD4⁺ T cells, appears to be the critical regulator of CD8⁺ T cell proliferation.

IL-2 has a very well established role in driving T cell proliferation (24). IL-2 is dispensable *in vivo* for the initial proliferation of responding CD8⁺ T cells, but is required to maintain proliferation of CD8⁺ T cells (25), and is especially important for accumulation of antigen-specific CD8⁺ T cells in non-lymphoid tissues (26). At very early times post-immunization (before day 6) the accumulation of unhelped Ad vector-elicited CD8⁺ T cells is equivalent to the accumulation of helped CD8⁺ T cells, but the relative proliferation rates then diverge. This very closely mimics the previously reported proliferative characteristics of CD8⁺ T cells that lack expression of CD25. Following vaccinia virus infection, the absence of CD4⁺ T cell-derived IL-2 substantially impairs accumulation of antigen-specific CD8⁺ T cells, and absence of high affinity IL-2R α expression on CD8⁺ T cells recapitulates the absence of CD4⁺ T cells (10). These data are highly consistent with the proposed model of how CD4⁺ T cells provide help to Ad vector-elicited CD8⁺ T cell responses. This suggests that CD4⁺ T cell-derived IL-2 is a generalizable mechanism by which CD4⁺ T cells provide help in the context of infections/immunizations where primary CD8⁺ T cell expansion requires CD4⁺ T cell help.

In addition to promoting the primary expansion of CD8⁺ T cell responses, CD4⁺ T cells are also critical for regulating the contraction of CD8⁺ T cell responses following Ad26 vector immunization. Such a role for CD4⁺ T cells has been reported following infections in other systems (6, 27, 28), but in these prior studies the timing of CD4⁺ T cell help was not investigated. Following Ad26 vector immunization, CD4⁺ T cell help is required for one month to regulate post-peak contraction of the CD8⁺ T cell response. After which CD8⁺ T cell responses were maintained steadily in the absence of CD4⁺ T cells. As with regulating expansion of CD8⁺ T

cells, there was a progressive long-term need for CD4⁺ T cell help. Interestingly, Ad vector immunization of IL-21R KO mice induces CD8⁺ T cell responses that exhibit more profound post-peak contraction than in WT mice (22). As CD4⁺ T cells are major producers of IL-21 (29), these data suggest that CD4⁺ T cell-derived IL-21 is likely one of the key factors for regulating CD8⁺ T cell contraction following Ad vector immunization. Further experiments will be required to directly test this hypothesis.

Finally, CD4⁺ T cell help can regulate the anamnestic potential of CD8⁺ T cells (6, 7, 30-33). A need for CD4⁺ T cell help in potentiating secondary proliferative capacity appears to be widely required for both “high-inflammation” and “low-inflammation” T cell stimuli, and thus, does not follow the demarcation observed for CD4⁺ T cell help and primary CD8⁺ T cell responses (8). The current model of how CD4⁺ T cell help regulates this process involves DCs acting as an intermediary between CD4⁺ T cells and CD8⁺ T cells (1, 31-35). In this model, CD4⁺ T cells license DCs by ligating the CD40 receptor expressed on DCs. In turn, this induces increased expression of CD70 on DCs, which interacts with its receptor, CD27, on CD8⁺ T cells. This CD70-CD27 signaling axis potentiates the CD8⁺ T cell to make IL-2, thereby enhancing their anamnestic potential. Based on these data, a model of CD4⁺ T cell regulation of anamnestic potential appears quite complete.

Unexpectedly, one report identified no defect in anamnestic potential of CD8⁺ T cells following Ad5 vector immunization when the cells were primed in MHC class II KO mice and transferred into a WT recipient prior to secondary antigen exposure (4). This study also reported secondary proliferation of CD8⁺ T cells in MHC class II KO mice upon antigen exposure even when adoptive transfers were not performed. However, these secondary effector cells displayed alterations in IFN- γ and TNF- α production. In my work, when CD4⁺ T cells were depleted on

day 5 post-Ad26 immunization the resultant CD8⁺ T cell response, despite exhibiting reduced primary expansion and alterations in phenotype, display normal anamnestic potential compared to cells from CD4⁺ T cell containing mice after adoptive transfer into naïve hosts. Unfortunately, the same experiment could not be performed using an earlier time of CD4⁺ T cell depletion due to limiting CD8⁺ T cell numbers. However, it appears that following Ad vector immunization, CD4⁺ T cell help is not required at priming for CD8⁺ T cells to expand normally upon secondary antigen exposure.

Interestingly, Ad vector-elicited CD8⁺ T cells from CD40L KO and CD40 KO mice have major defects in secondary expansion upon antigen re-exposure even if the cells are transferred into a WT recipient mouse prior to the boosting immunization. It has been reported that DCs and CD8⁺ T cells can signal directly via the CD40 pathway (36). Thus, while this pathway is important for regulating CD8⁺ T cell anamnestic potential following Ad vector immunization, it does not appear to involve CD4⁺ T cells. Further experiments will be required to confirm that CD8⁺ T cell anamnestic potential following Ad vector immunization does not require CD4⁺ T cells, and to identify exactly how CD40 is involved in this process.

The results presented in this thesis, when synthesized with prior published work, describe a very detailed picture of the role of CD4⁺ T cell help in expansion and contraction of the primary CD8⁺ T cell response following Ad vector immunization, as well as the apparent lack of a role for CD4⁺ T cells in regulating anamnestic potential. These data demonstrate the early and prolonged requirement for CD4⁺ T cell help in the expansion of the primary CD8⁺ T cell response, which appears to be mediated largely by IL-2. Additionally, a prolonged need for CD4⁺ T cell help is observed to maintain CD8⁺ T cell numbers during the post-priming contraction phase. In conclusion, this work substantially enhances our fundamental

understanding of how Ad vector-elicited CD8⁺ T cell responses are regulated, and this may have relevance for the use of Ad vectors in the clinical setting.

Timing of CD4⁺ T cell help to B cells

CD4⁺ T cells have a well-established role in promoting antibody responses (37, 38). In the context of antibody responses, CD4⁺ T cell help is provided primarily by a subset of CD4⁺ T cells known as T follicular helper (T_{FH}) cells, and one of the major functions of these cells is to initiate and maintain germinal center B cell responses (39). As part of this process, CD4⁺ T cells provide signals to germinal center B cells allowing these cells to undergo somatic hypermutation and ultimately leave the germinal center and become antibody-secreting plasma cells (39). A critical pathway involved in these processes is CD40, where CD40L expressed on CD4⁺ T cells binds to CD40 expressed on B cells to provide the relevant positive signals (40). This group thus utilized antibody-mediated blockade of CD40 signaling to determine the timing of CD4⁺ T cell help. Following immunization with a haptenated-protein formulated in alum, the administration of α CD40L blocking antibody from day -1 to 3 post-immunization abolished the germinal center B cell responses and reduced serum antigen-specific antibody titers, while α CD40L blocking antibody treatment begun on day 6 post-immunization had no effect on antigen-specific antibody titers (40). Subsequent detailed microscopy analysis has observed prolonged interactions between T_{FH} cells and B cells following immunization with a haptenated-protein (41). Thus, there is strong data showing a multi-day need for CD4⁺ T cell help for the optimal development of antibody responses following protein immunization.

When the role and timing of CD4⁺ T cell help was examined following Ad vector immunization many parallels were observed. CD4⁺ T cell help is required for transgene-specific

antibody responses, CD40 signaling is a critical signaling pathway, and CD4⁺ T cells modulate germinal center B cell responses. Furthermore, longitudinal depletion of CD4⁺ T cells following immunization identified that CD4⁺ T cell help is needed for between 10 and 14 days and that CD4⁺ T cell help is most required early post-immunization and less so by day 10. Thus, the role of CD4⁺ T cell help in Ad vector immunization appears analogous to that observed in protein immunization, with minor differences in timing, which likely reflects differences in the overall kinetics of the Ad vector-induced immune response.

While depletion of CD4⁺ T cells at the time of Ad vector immunization completely ablates the development of transgene antigen-specific antibody responses at day 30 post-immunization, this does not result in a permanent defect in the antigen-specific antibody responses. When CD4⁺ T cells are depleted transiently using α CD4 antibody, the antigen-specific antibody responses are simply delayed until the α CD4 antibody is cleared from the animal and CD4⁺ T cell compartment recovers. This phenomenon was also observed following immunization with a soluble protein formulated in alum adjuvant. Such an observation has not been previously reported, and several other studies have demonstrated long-term tolerance to the original immunizing antigen if CD4⁺ T cells were transiently depleted by monoclonal α CD4 antibody administration (42-46).

I hypothesize that long-term antigen persistence and the development of antigen-specific CD4⁺ T cell responses following transient CD4⁺ T cell depletion are two necessary factors for these delayed antibody responses to occur. Transient depletion of CD4⁺ T cells in the context of chronic LCMV infection results in persistent antigen expression (47). However, antigen-specific CD4⁺ T cell responses do not develop (47, 48). But if naive antigen-specific CD4⁺ T cells are transferred into chronically LCMV infected mice, the CD4⁺ T cells become primed and LCMV-

specific antibody responses develop (46). Using these data as a template for how delayed antibody responses can be induced, it appears that persistent antigen (to prime recovering CD4⁺ T cells) and antigen-specific CD4⁺ T cells are required. Ad vector immunization has persistent antigen expression when CD4⁺ T cells are absent ((4) and Fig. 3.S1), which would allow for T cell priming. Further experiments will be required to confirm the presence of antigen-specific CD4⁺ T cells following transient CD4⁺ T cell depletion in the context Ad vector immunization, as hypothesized. In summary, these data demonstrate that antibody responses can be elicited by Ad vector or alum-adjuvanted protein immunization at a time markedly distinct from the time of immunization by modulating CD4⁺ T cell help. An ability to temporally separate the time of antibody induction from the time of antigen exposure has not been previously reported. This observation likely has substantial clinical relevance as it demonstrates that therapeutic efforts to increased CD4⁺ T cell frequency and/or function, even after the time of immunization, can dramatically improve Ad vector vaccine immunogenicity. Hopefully future clinical successes will corroborate this hypothesis.

ABERRANT DIFFERENTIATION OF CD8⁺ T CELLS IN THE ABSENCE OF CD4⁺ T CELLS

The second major finding of this thesis is that following Ad vector immunization, CD4⁺ T cells play a critical role in proper differentiation of functional CD8⁺ T cell responses, in addition to regulating the magnitude of the responses, as discussed in the previous section. The data presented in chapter 2 provide initial description of the phenotypic abnormalities of CD8⁺ T cells in the absence of CD4⁺ T cells. The experiments performed in chapter 3 describe the phenotypic abnormalities of CD8⁺ T cells primed without CD4⁺ T cell help in more detail, and demonstrate

that functional defects are also present. Furthermore, the data in chapter 3 clearly demonstrate that the mechanisms of CD4⁺ T cell help are multi-factorial, and presents data for a subset of these processes. A role for CD4⁺ T cells in promoting effector CD8⁺ T cell differentiation is not well established, as prior reports have observed no alteration in effector differentiation or increased effector differentiation in the absence of CD4⁺ T cells (49-51). Thus, the work presented here identifies a new aspect of CD4⁺ T cell regulation of CD8⁺ T cell responses.

Functional CD8⁺ T cell responses: effector and memory subsets

CD8⁺ T cells differentiate into two highly functional subsets upon priming with an acutely cleared pathogen or immunization regimen: 1) effector cells, which have robust cytotoxic and cytokine producing potential, and 2) memory cells, which have increased long-term survival and anamnestic capacity (52). In mice, short-lived effector cells can be identified using cell surface markers as KLRG1^{hi}CD127^{lo}, while long-lived memory precursor cells have reciprocal expression of these two markers (KLRG1^{lo}CD127^{hi}) (53, 54). Following Ad vector immunization, a sizeable fraction of long-lived memory cells that are KLRG1^{hi}CD127^{hi} develop at late times post-immunization (Fig. 2.6 (12)), and such cells appear to have intermediate characteristics of memory and effector cells (52). The regulation of effector versus memory cell is controlled by a number of transcription factors and transcriptional repressors, including the effector promoting T-bet, Blimp-1, and Id2, and the memory promoting Eomes, Bcl-6, Id3, and TCF1 (55-61).

In the absence of CD4⁺ T cells Ad vector-elicited CD8⁺ T cells have a defect in differentiation to an effector state. Unhelped CD8⁺ T cells do not acquire an effector phenotype as evidenced by decreased expression of KLRG1. Defects in canonical effector function are also

observed, as unhelped CD8⁺ T cells are less cytotoxic and produce fewer cytokines. These functional defects correspond to reduced expression of T-bet and Eomes. Eomes expression is positively regulated by IL-2, and its expression is required for optimal cytotoxic function (62). Following Ad vector immunization, absence of CD8⁺ T cell-intrinsic high affinity IL-2 signaling recapitulates the reduced cytotoxic phenotype of unhelped CD8⁺ T cells, and administration of rIL-2 to CD4⁺ T cell depleted mice can rescue the cytotoxic phenotype of unhelped CD8⁺ T cells. In the absence of CD4⁺ T cells Ad vector-elicited CD8⁺ T cells fail to acquire effector phenotype and function, and this appears to be due, in part, to the absence of CD4⁺ T cell-derived IL-2.

CD8⁺ T cells primed by Ad vector immunization in the absence of CD4⁺ T cells have increased phenotypic characteristics of memory cells, but this does not appear to correspond to increased functional potential. Unhelped CD8⁺ T cells express elevated levels of CD127 as well as CD62L, a canonical marker of central memory cells (63). However, unhelped CD8⁺ T cells express less CD27, another canonical marker of memory cells (64). These cells also express less Eomes and Bcl6, which are both transcriptional regulators that promote long-term memory differentiation. Central memory cells have greater anamnestic potential than other memory cells (63). If unhelped CD8⁺ T cells really were enriched for central memory cells based on increased expression of CD62L, then unhelped CD8⁺ T cells should have increased proliferative capacity upon secondary antigen exposure compared to helped CD8⁺ T cells. However, when cells are adoptively transferred to normalize cell number prior to boosting immunization, Ad vector-elicited CD8⁺ T cells that have only received help for 5 days and have elevated expression of CD62L have equivalent anamnestic expansion as helped CD8⁺ T cells. Other aspects of memory capacity, such as proliferation in response to homeostatic cytokines, were not compared between helped and unhelped cells, but this would be an interesting area of future investigation to more

fully understand the altered characteristics of unhelped CD8⁺ T cells. In sum, it appears that despite some phenotypic characteristics suggesting increased memory properties of unhelped CD8⁺ T cells, these cells do not actually exhibit increased memory functionality by the crucial measure tested.

CD8⁺ T cells primed by Ad vector immunization have profound defects in effector phenotype and function. These cells also have atypical expression of memory markers, but do not have increased memory cell functionality. Instead, unhelped CD8⁺ T cells are highly dysfunctional and share many functional and transcriptional characteristics of several hypo-functional T cell differentiation states.

Hypo-functional CD8⁺ T cell responses: exhaustion, tolerance, and anergy

In addition to the functional T cell differentiation states of effector cells and memory cells, there are three well defined differentiation states of T cell hypo-functionality: exhaustion, tolerance, and anergy (65, 66). Given the clear defect in differentiation of unhelped CD8⁺ T cells towards a functional effector state, it is important to understand how, if at all, these abnormal unhelped CD8⁺ T cells relate to one of these well-described hypo-functional states.

Exhaustion occurs in the setting of persistent antigen exposure, such as chronic infection or cancer (67). Exhausted CD8⁺ T cells are characterized by a distinct expression pattern of cell surface molecules with substantial upregulation of multiple inhibitory receptors (68-72). Exhausted cells also have major defects in cytokine production and altered cytotoxic capacities (70, 73, 74). Unhelped Ad vector-elicited CD8⁺ T cell responses share many of these phenotypic and functional characteristics with exhausted cells. The absence of CD4⁺ T cells results in increased expression of inhibitory receptors on Ad vector-elicited CD8⁺ T cells. Unhelped CD8⁺

T cells have defects in cytokine production and cytotoxicity that are very similar to those reported in exhausted cells. Although it is now clear that elevated inhibitory receptor expression is not causative of exhaustion (75), modulation of inhibitory receptor signaling can improve exhausted T cell functionality (69, 70, 76). Similarly, blockade of PD-1 signaling during priming of CD8⁺ T cells in the absence of CD4⁺ T cells partially prevents this impaired effector differentiation. Exhausted CD8⁺ T cells can be reinvigorated *in vivo* by treatment with rIL-2 (77), and rIL-2 administration can partially rescue effector functionality of unhelped Ad vector-elicited CD8⁺ T cells. In sum, unhelped Ad vector elicited CD8⁺ T cells share many phenotypic and functional traits with exhausted CD8⁺ T cells, including being functionally regulated by inhibitory receptors.

Exhausted CD8⁺ T cells have a unique transcriptional profile and an altered transcriptional network compared to either effector or memory T cells (71, 78). This altered transcriptional network includes altered expression of T-bet and Eomes (79). The transcriptional signature of exhaustion reported in two studies (71, 80) is shared with unhelped CD8⁺ T cells. However, specific longitudinal network analysis has not been performed on unhelped Ad vector-elicited CD8⁺ T cells, and this would be an ideal area of future investigation. Alterations in T-bet and Eomes expression, which broadly represent the perturbations in exhausted cells, were observed in unhelped CD8⁺ T cells (data not shown), but functional lineage relationship analysis needs to be performed before it can be determined if altered T-bet and Eomes signaling are functionally important for the dysregulated differentiation of unhelped CD8⁺ T cells. A very clear signal for atypical NFAT signaling was observed in unhelped CD8⁺ T cells (Fig. 3.3). AP-1-independent NFAT signaling drives expression of a distinct subset of genes that includes exhaustion genes (81), and these AP-1-independent NFAT target genes, including exhaustion

genes, are significantly enriched in unhelped CD8⁺ T cells. In addition to sharing functional and phenotypic abnormalities with exhausted CD8⁺ T cells, unhelped CD8⁺ T cells share transcriptional signatures with exhausted CD8⁺ T cells. Future work will be required to determine to what degree this reflects shared alterations in transcriptional networks, and to identify the key transcription factors that regulate this unhelped CD8⁺ T cell phenotype.

T cell exhaustion occurs due to exposure of cells to persistent antigen (82-84), and elevated type I interferon signaling plays a critical role (85, 86). However, neither of these processes are major factors in the dysfunction of unhelped CD8⁺ T cells (Fig. 3.S4 and data not shown). Additionally, exhaustion is a progressive phenomenon wherein the cells become more exhausted over time (73, 74). This means that exhaustion can be prevented if CD8⁺ T cells are removed from the exhausting environment early enough (87). In contrast, unhelped CD8⁺ T cells display functional abnormalities immediately upon priming, and these cells were irreversibly committed to this aberrant differentiation program by day 3 post-immunization. While unhelped dysfunctional CD8⁺ T cells share functional, phenotypic, and transcriptional characteristics with exhausted CD8⁺ T cells, these data suggest the underlying causative mechanisms are largely distinct.

Similar to immune exhaustion, tolerance is a progressive process wherein cells lose functionality over time. In peripheral tolerance, self-reactive T cells that have escaped negative thymic selection are driven to a non-functional state. This program is maintained by continuous exposure to self-antigen (88). Peripheral tolerance can also be induced against foreign antigens by repeated peptide administration in the absence of sufficient stimulatory signals (17, 89, 90). This state of peripheral tolerance has recently been demonstrated to involve a distinct transcriptional program from other states of T cell hypo-functionality (91). This genetic signature

of peripheral tolerance was used to determine whether CD8⁺ T cells primed by Ad vector immunization without CD4⁺ T cell help express a transcriptional signature of tolerance. The tolerance gene signature is not significantly enriched in CD8⁺ T cells primed without CD4⁺ T cell help (data not shown). Thus, at the transcriptional level it does not appear that CD8⁺ T cells primed without CD4⁺ T cell help have been tolerized.

The final well-described hypo-functional state is anergy, which occurs when T cells undergo incomplete priming. It is now understood that the term anergy actually describes a large number of mechanistically distinct states of incomplete priming and immediate dysfunction (92). The unifying theme of these mechanistically distinct processes is that incomplete T cell priming due to the absence of one or more critical stimulatory signal is the cause, and that anergy is a process of immediate hypo-functionality. Transcriptional profiling of CD4⁺ T cells anergized *in vitro* by TCR stimulation in the absence of CD28 co-stimulation has provided a transcriptomic understanding of how this specific type of anergy is regulated (93). This anergic transcriptional signature is significantly enriched in unhelped CD8⁺ T cells following Ad vector immunization. As discussed above, the transcriptional signature of AP-1-independent NFAT signaling is enriched in unhelped CD8⁺ T cells. AP-1-independent NFAT target genes also include genes upregulated in anergy (81), and the gene signature for these anergy-related AP-1 independent NFAT target genes is enriched in unhelped CD8⁺ T cells. Thus, non-canonical NFAT signaling appears to be an important modulator of the anergic transcriptional profile observed in unhelped CD8⁺ T cells.

However, perturbation of CD28-mediated signaling does not appear be the reason for anergy of unhelped CD8⁺ T cells induced by Ad vector immunization. Absence of CD28 signaling completely abolished the CD8⁺ T cell response following Ad vector immunization,

consistent with a prior report (94). Additionally, the absence of CD4⁺ T cells does not alter the upregulation of CD80 and CD86 on dendritic cells in the draining LNs following Ad vector immunization (data not shown). Thus, the absence of co-stimulation and the absence of CD4⁺ T cells are not equivalent in this system. However, as transcriptional signatures for anergy induced by other experimental protocols do not exist, it is possible that the transcriptional signature of anergy is conserved across anergy induced by different mechanisms. This would potentially explain why unhelped CD8⁺ T cells express the transcriptional signature of anergy induced by absence of co-stimulation despite all evidence suggesting co-stimulation is unimpaired when CD4⁺ T cells are absent. More detailed analysis of the transcriptional processes of anergy may clarify this possibility.

CD8⁺ T cells can undergo a specific form of anergy termed split-anergy (or activation-induced non-responsiveness), where CD8⁺ T cells fail to receive the complete array of priming signals and immediately enter a hypo-functional state (95). This split-anergy is characterized by normal IFN- γ production and cytotoxic function, but impaired proliferative capacity (96). Unhelped CD8⁺ T cells do not appear to be in a state of split-anergy as these cells have major defects in proliferation and also have dramatic impairments in IFN- γ production and cytotoxic function. Administration of rIL-2 has been demonstrated to reverse anergy, including split-anergy (97, 98). In our system, administration of rIL-2 corrects the dysfunctional CD8⁺ T cell phenotype, and absence of IL-2 signaling is sufficient to reproduce some facets of the observed T cell dysfunction. The responsiveness of unhelped CD8⁺ T cells to IL-2 treatment is in good agreement with the observed transcriptional signature of anergy. The dysfunction of unhelped CD8⁺ T cells we observe can be defined as anergy given the immediacy of the impairment and a

common transcriptional signature, but the functionality and phenotype of the responding cells are distinct from the previously reported states of anergy.

In sum, unhelped CD8⁺ T cells exhibit a dysfunctional profile that is best described as a combination of exhaustion and anergy. Transcriptional profiles of both hypo-functional states are observed in unhelped CD8⁺ T cells, likely due to increased non-canonical NFAT signaling. The immediacy of the dysfunction mirrors anergy, while the decreased functionality and increased expression of inhibitory receptors mirrors T cell exhaustion. Most importantly, CD4⁺ T cells have not been previously reported to play a major role in regulating CD8⁺ T cell effector differentiation, but following Ad vector immunization, CD4⁺ T cells clearly play a major role in preventing T cell dysfunction.

The role of CD4⁺ T cell help in influencing primary CD8⁺ T cell functionality in other models

CD4⁺ T cells have well-established roles in regulating primary CD8⁺ T cell expansion, maintenance, and anamnestic potential. However, a role for CD4⁺ T cells in regulating CD8⁺ T cell effector differentiation is not well established. Two studies have observed increased differentiation of CD8⁺ T cells towards an effector phenotype following infection with LCMV (50) or vaccinia virus (51) in the absence of CD4⁺ T cells. However, a separate study found no alternation in effector phenotype of CD8⁺ T cells primed by vaccinia virus in the absence of CD4⁺ T cells (49). In contrast, following Ad vector immunization, CD4⁺ T cells are absolutely critical for acquisition of effector phenotype and functions by CD8⁺ T cells, and appear to regulate all aspects of CD8⁺ T cell functionality.

The hyper-effector phenotype observed in unhelped CD8⁺ T cells following LCMV infection is due to elevated expression of the transcription factor T-bet (50). By contrast,

unhelped CD8⁺ T cells elicited by Ad vector immunization have decreased expression of T-bet. T-bet expression can be driven by inflammatory signals (62). It has been discussed above that the need for CD4⁺ T cell help to drive primary effector differentiation in different experimental systems appears to depend on whether the infection/immunization induces strong inflammation (8). This same demarcation appears to apply with regards to the requirement for CD4⁺ T cell help and effector differentiation of CD8⁺ T cells. High inflammation environments, such as LCMV infection, can induce effector-lineage promoting transcription factors independent of CD4⁺ T cells, while low inflammation environments require CD4⁺ T cell-derived signals, IL-2 being one factor, to induce expression of these transcription factors. Consistent with this hypothesis, replication-incompetent NYVAC poxvirus vector induced CD8⁺ T cell responses, like Ad vector-elicited responses, had decreased effector phenotype, and these vectors induce very modest innate inflammation (99). In summary, the ability of CD8⁺ T cells to acquire effector functionality is dependent on CD4⁺ T cell help following priming with multiple replication-incompetent viral vectors. Further experiments will be required to determine how widespread is the need for CD4⁺ T cell help to promote effector CD8⁺ T cell differentiation.

SUMMARY

In conclusion, the data presented in this thesis identify the key role of CD4⁺ T cells in promoting optimal cellular and humoral immune responses following adenovirus vector immunization. These data thoroughly demonstrate for the first time the time-dependent effects of CD4⁺ T cell help. With regards to CD4⁺ T cell help to CD8⁺ T cells, these data demonstrate a previously unappreciated role for CD4⁺ T cells in promoting functional cytotoxic effector differentiation of CD8⁺ T cells. CD4⁺ T cell help to CD8⁺ T cells is a multifactorial process that involves IL-2,

regulation of NFAT signaling, and other to-be-identified factors. Unexpectedly, in the context of antibody responses, CD4⁺ T cell help can be provided at a time temporally distinct from the time of immunization. Such a temporal separation of CD4⁺ T cell help from the time of antigen exposure has not been previously reported. Thus, while CD4⁺ T cell help is critical for both cellular and humoral immune responses, the timing and mechanisms of help are distinct. These data have major implications for our understanding of the process that govern vaccine-elicited CD8⁺ T cell and antibody responses, and for understanding how the processes that regulate vaccine-elicited responses are similar or different from responses induced by natural infection. This knowledge can be applied to the development of improved, rationally designed, next-generation vaccines.

FUTURE DIRECTIONS

Identifying additional pathways involved in CD4⁺ T cell regulation of CD8⁺ T cells

The major outstanding question regarding the role of CD4⁺ T cells in the regulation of Ad vector-elicited CD8⁺ T cell responses is: in addition to IL-2, what other pathways are involved? Three complimentary approaches can be taken to begin to address this question.

First, it should be determined definitively whether CD4⁺ T cells provide help to Ad vector-elicited CD8⁺ T cells by providing activating signals to dendritic cells, as has been described in other systems (31-33, 35, 100). Data in this thesis demonstrates that CD40 signaling is not a major mechanism of CD4⁺ T cell help in this system, but there are other pathways by which CD4⁺ T cells can activate dendritic cells. To formally assess a role for CD4⁺ T cell-mediated dendritic cell maturation a mixed bone marrow chimera experiment should be performed. As described in (35), wild type mice will be irradiated and reconstituted with a 50/50

mixture of MHC class I KO and MHC class II KO bone marrow. Upon reconstitution, these mice will be capable of mounting both CD4⁺ T cell and CD8⁺ T cell responses, but a single dendritic cell cannot present cognate antigen to both a CD4⁺ T cell and a CD8⁺ T cell (since any given dendritic cell will lack either MHC class I or MHC class II). Thus, this experimental setup allows for definitive clarification as to whether CD4⁺ T cells provide cell-cell contact-dependent maturation signals to dendritic cells following Ad vector immunization. This knowledge will allow for the exclusion, or inclusion, of a large number of possible mechanisms of CD4⁺ T cell help.

Second, transcriptional profiling should be performed on day 4 (or 5 if cell numbers are limiting) post-immunization antigen-specific CD8⁺ T cells from α CD4 antibody treated or untreated control mice. Gene set enrichment analysis and pathway analysis can be performed, as in Fig 3.3, on these data to identify possible pathways that are differentially activated when CD4⁺ T cells are present or absent. Synthesizing these data with the data generated from the first experiment should identify candidate pathways of CD4⁺ T cell help. These candidate pathways can then be validated in the following experiment.

Third, candidate pathways of CD4⁺ T cell help identified in experiments one and two should be validated experimentally. If gene knockout animals for a candidate pathway are available then a given pathway can be tested easily. However, a more systematic approach is preferred. Specifically, the experimental technique of adoptive transfer of antigen-specific CD4⁺ T cells into thymectomized mice, as in Fig. 3.4, to experimentally control the characteristics of CD4⁺ T cells can be utilized. The antigen-specific CD4⁺ T cells can have specific genes knocked down by retroviral shRNA vectors prior to adoptive transfer. Alternatively, CRISPR-Cas9-mediated gene deletion could be utilized. Such an approach would allow for a reasonably high-

throughput setup to rapidly screen a number of putative pathways. Furthermore, this technique would demonstrate the specific role for a given pathway on CD4⁺ T cells, which is the critical factor for demonstrating a given pathway is a direct mechanism of CD4⁺ T cell help. Thus, this experimental technique represents a robust means to experimentally validate additional mechanisms of CD4⁺ T cell help.

Together, these data will provide additional mechanistic insights into how CD4⁺ T cells provide help to CD8⁺ T cells following Ad vector immunization.

Enhancing our understanding of delayed antibody responses

To better understand how delayed antibody responses develop following transient depletion of CD4⁺ T cells, the following experiments should be performed.

First, the nature of CD4⁺ T cell help following transient depletion of CD4⁺ T cells should be better described. It is presumed that following transient depletion of CD4⁺ T cells in the context of Ad vector or alum-adjuvanted recombinant protein immunization, the recovering CD4⁺ T cell population contains activated antigen-specific CD4⁺ T cells. This hypothesis should be confirmed, and the necessity of antigen-specific cells should be demonstrated. To confirm the presence of antigen-specific CD4⁺ T cells intracellular cytokine staining (ICS) after cognate peptide stimulation can be performed. Additionally, T_{FH} phenotype cells (PD-1⁺ CXCR5^{hi}) can be identified by surface marker analysis, and these cells should only be present in antigen-experienced animals. To determine whether antigen-specific CD4⁺ T cells are necessary for these delayed antibody responses to develop, antigen-specific CD4⁺ T cells or irrelevant antigen CD4⁺ T cells can be adoptively transferred into thymectomized mice on day 30 post-immunization. As thymectomized mice do not develop delayed antibody responses (Fig. 4.4), this experimental

approach allows for the specific investigation of the need for antigen-specific CD4⁺ T cells in this process. These experiments will test the hypothesis that the development of a delayed antibody response requires the involvement of antigen-specific CD4⁺ T cells.

Second, how persistent antigen is involved in the induction of delayed antibody responses remains to be determined. In the absence of CD4⁺ T cells, transgene expressing cells are abundant for months post-immunization (Fig. 3.S1), or following immunization with protein formulated in adju-phos the protein may be retained within an antigen depot. Therefore, free antigen may be responsible for the induction of delayed antibody responses. Alternatively, follicular dendritic cells (FDCs) can retain antigen in the form of immune complexes for extended periods (101), and thus free soluble antigen may be dispensable for this process. To test these two possibilities several experiments can be performed. To determine if antigen depoting is necessary for this phenomenon, protein can be formulated in a non-depotting adjuvant (ie. CpG) or a depotting adjuvant (ie. CpG + emulsigen) prior to immunization. If depotting is required then only that formulation should induce a delayed antibody response. Alternatively, a transgene-silencing Ad vector, as used in Fig. 3.S4, could be constructed expressing SIV Env. To directly test the role of FDCs in the induction of delayed antibody responses, mice that lack fully functional FDCs due to specific gene knockout can be utilized. These experiments will provide greater insights into the exact nature of the antigen that is required for the induction of a delayed antibody response.

Third, the exact nature and timing of B cell priming in the context of a delayed antibody response should be investigated. The major outstanding question is: are B cells primed at the time of immunization and remain quiescent until CD4⁺ T cell help is provided, or are B cells newly primed when CD4⁺ T cells recover? To directly test this, all B cells can be transiently

depleted via anti-CD20 antibody after immunization. If transient depletion of B cells prior to the recovery of CD4⁺ T cells prevents the development of a delayed antibody response then B cell priming at the time of immunization can be presumed to be critical for this process to occur. As an additional set of experiments, T cell-independent B cell responses can be assessed by antigen-specific IgM ELISPOT to detect the presence and activation of non-class-switched B cells at various times prior to and after the recovery of CD4⁺ T cells. Finally, if B cell activation is observed at the time of immunization in the absence of CD4⁺ T cells then fate tracking can be performed to determine if these B cells are the ones that give rise to the delayed antibody response after recovery of CD4⁺ T cells. These experiments will better define when B cells get activated and primed for the induction of a delayed antibody response.

Collectively, these three areas of investigation will dramatically enhance our understanding of the processes that regulate the induction of a delayed antibody response following transient depletion of CD4⁺ T cells.

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Appendix: Supplemental Figures

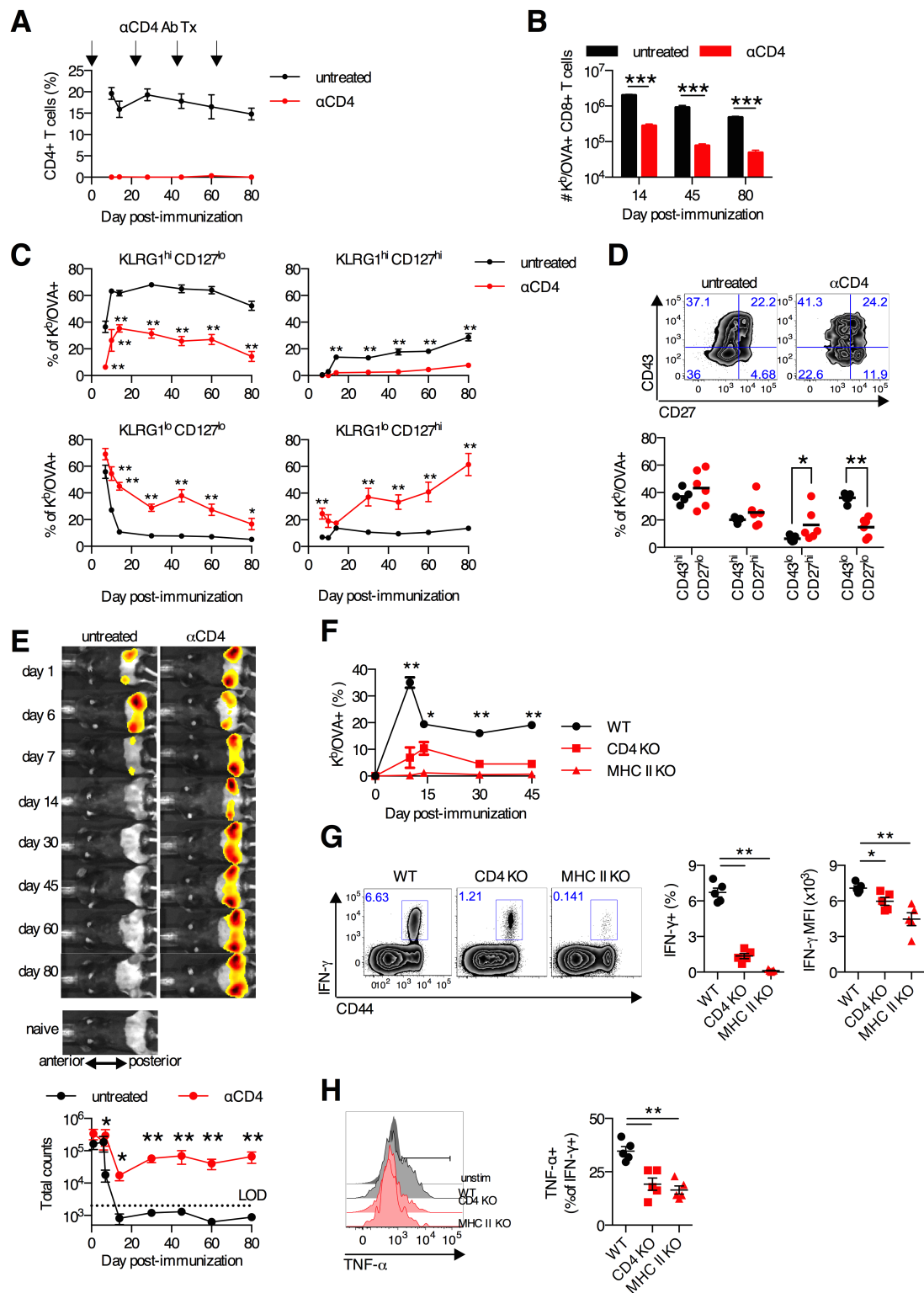


Figure 3.S1. Long-term dysfunctional phenotype of CD8⁺ T cells primed in the absence of CD4⁺ T cells.

Figure 3.S1 (Continued)

(A-E) C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10^9 vp of Ad5-SIINFEKL-Luc. (A) Frequency of CD4⁺ T cells in the blood. (B) Absolute number of K^b/OVA⁺ CD8⁺ T cells in the spleen. (C) Expression of KLRG1 and CD127 on K^b/OVA⁺ CD8⁺ T cells in the blood. (D) Expression of CD27 and CD43 on K^b/OVA⁺ CD8⁺ T cells in the spleen day 14 post-immunization. (E) *in vivo* luciferase transgene expression in a single representative animal per group over time or group average as quantified by IVIS. (F-H) C57BL/6, CD4 KO, or MHC II KO mice were immunized intramuscularly with 10^9 vp of Ad5-SIINFEKL-Luc. (F) Frequency of K^b/OVA⁺ cells as a fraction of CD8⁺ cells in the blood. (G) Frequency of IFN- γ -secreting CD8⁺ T cells in the spleen on day 45 following *ex vivo* OVA₂₅₇₋₂₆₄ peptide stimulation and MFI of IFN- γ in IFN- γ ⁺ cells. (H) TNF- α expression by IFN- γ ⁺ CD8⁺ T cells following *ex vivo* stimulation with OVA₂₅₇₋₂₆₄ peptide. (A, C, D) Data are representative of three experiments (n=5-6/group per experiment). (B) Data are pooled from three experiments (n=5/group per experiment). (E-H) Data are representative of two experiments (n=5/group per experiment). Mean \pm SEM are shown. ***, P<0.001; **, P<0.01; *, P<0.05; Mann-Whitney U test.

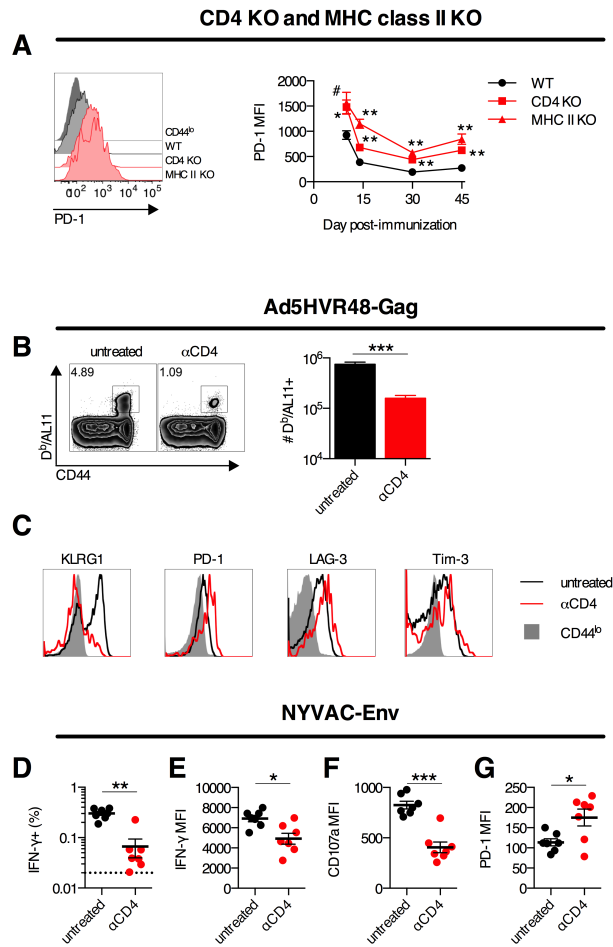


Figure 3.S2. Elevated expression of inhibitory receptors on CD8⁺ T cells in multiple experimental settings of CD4⁺ T cell deficiency. (A) C57BL/6, CD4 KO, or MHC II KO mice were immunized intramuscularly with 10⁹ vp of Ad5-SIINFEKL-Luc. Frequency of K^b/OVA⁺ cells as a fraction of CD8⁺ cells in the blood. Data are representative of two experiments (n=5/group per experiment). (B, C) C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10⁹ vp of Ad5HVR48-Gag. (B) Frequency of D^b/AL11⁺ CD8⁺ T cells in the spleen on day 14 post-immunization. (C) KLRG1, PD-1, Lag-3, and Tim-3 expression on D^b/AL11⁺ CD8⁺ T cells in the spleen. Data are pooled (B) or representative (C) of six experiments (n=5-7/group per experiment). (D-G) C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10⁶ pfu of NYVAC-Env (n=7/group). (D) Frequency of IFN- γ ⁺ Env-specific CD8⁺ T cells in the spleen on day 14. (E) Per cell production of IFN- γ by IFN- γ ⁺ CD8⁺ T cells as measured by mean fluorescence intensity (MFI). (F) CD107a expression by IFN- γ ⁺ CD8⁺ T cells upon Env peptide pool stimulation. (G) PD-1 expression on IFN- γ ⁺ CD8⁺ T cells. Mean \pm SEM are shown. **, P<0.01; *, P<0.05; #, P=0.06; Mann-Whitney U test.

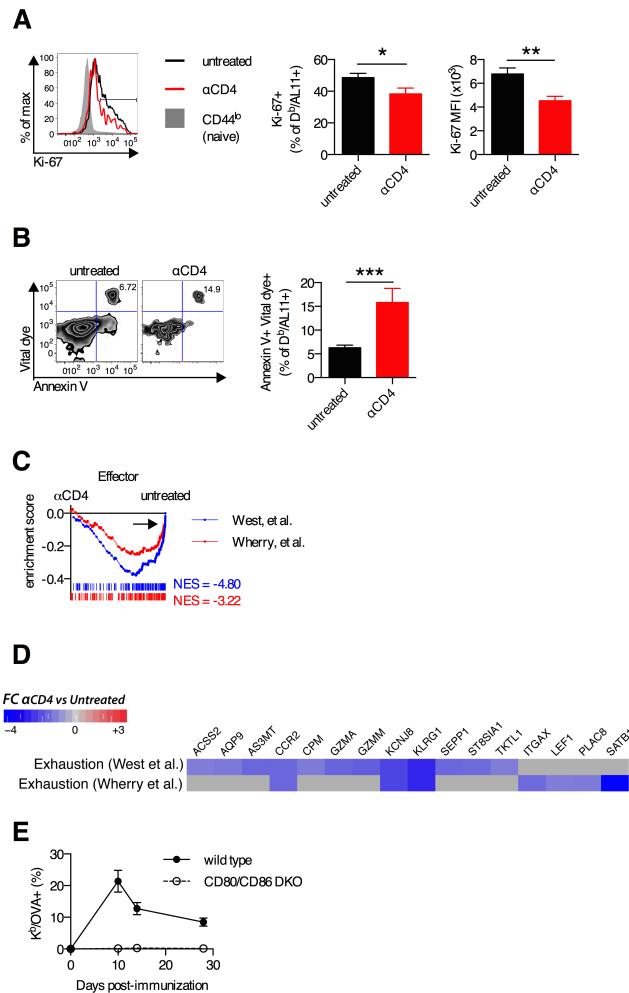


Figure 3.S3. Experimental validation of gene expression profiling findings. (A-D) C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10^9 vp of Ad5HVR48-Gag. (A) Ki-67 expression by D^b/AL11⁺ CD8⁺ T cells in the spleen on day 14 post-immunization. (B) Frequency of Annexin V⁺ vital dye⁺ apoptotic D^b/AL11⁺ CD8⁺ T cells in the spleen on day 14 post-immunization. (C) Enrichment of the effector gene signature from [GSE9650 (24), GSE30962 (23)] in untreated control D^b/AL11⁺ CD8⁺ T cells. (D) Enriched genes from the leading edge analysis of (C). (E) C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10^9 vp of Ad5-SIINFELK-Luc. Frequency of K^b/OVA⁺ CD8⁺ T cells in the blood was assessed. (A,B) Data are pooled from two experiments (n=5-7/group per experiment). (C,D) Data are from one experiment. (E) Data are representative of two experiments (n=5/group per experiment). Mean \pm SEM are shown. **, P<0.01; *, P<0.05; #, P=0.06; Mann-Whitney U test.

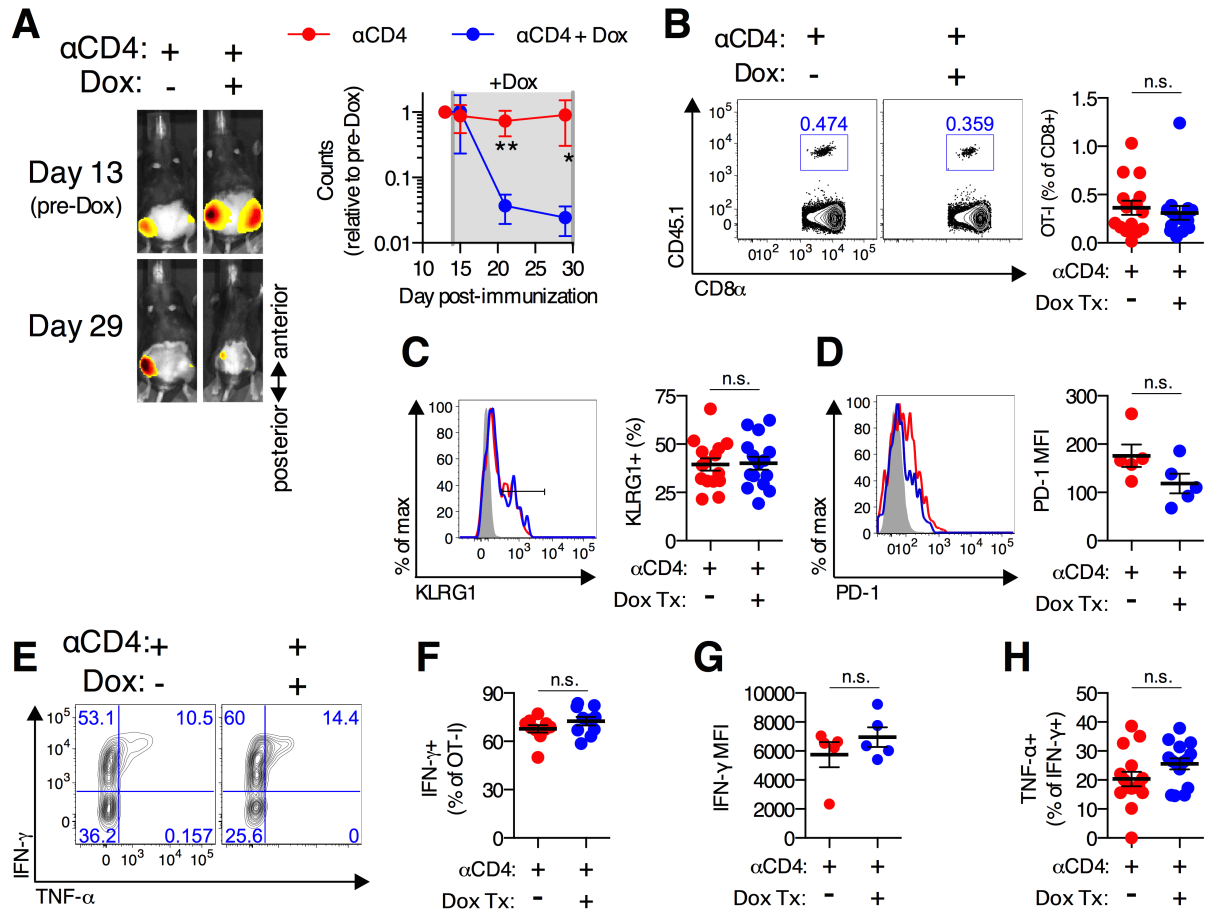


Figure 3.S4. Reduced transgene clearance in the absence of CD4⁺ T cells does not alter CD8⁺ T cell responses. 500 naïve OT-I CD8⁺ T cells were transferred into CD45.1⁺ congenic recipient mice. Mice were treated with α CD4 antibody and immunized intramuscularly with 10¹⁰ vp of Ad5-tTA-SIINFEKL-Luc. **(A)** *in vivo* luciferase transgene expression in a single representative animal per group over time and group average as quantified by IVIS. **(B)** Representative plots and group average of CD45.1⁺ donor OT-I CD8⁺ T cells. **(C)** KLRG1 expression on CD45.1⁺ OT-I CD8⁺ T cells. **(D)** PD-1 expression on CD45.1⁺ OT-I CD8⁺ T cells. **(E)** Representative plot of expression of IFN- γ and TNF- α on CD45.1⁺ OT-I CD8⁺ T cells following OVA₂₅₇₋₂₆₄ peptide stimulation. **(F)** Fraction of CD45.1⁺ OT-I CD8⁺ T cells that produce IFN- γ upon OVA₂₅₇₋₂₆₄ peptide stimulation. **(G)** Mean fluorescence intensity (MFI) of IFN- γ ⁺ OT-I CD8⁺ T cells. **(H)** TNF- α production by IFN- γ ⁺ OT-I CD8⁺ T cells. Data are pooled from three experiments (**B**, **C**, **H**), pooled from two experiments (**F**), or representative of three experiments (**A**, **D**, **E**, **G**). N=5/group per experiment. Mean \pm SEM are shown. **, P<0.01; *, P<0.05; Mann-Whitney U test.

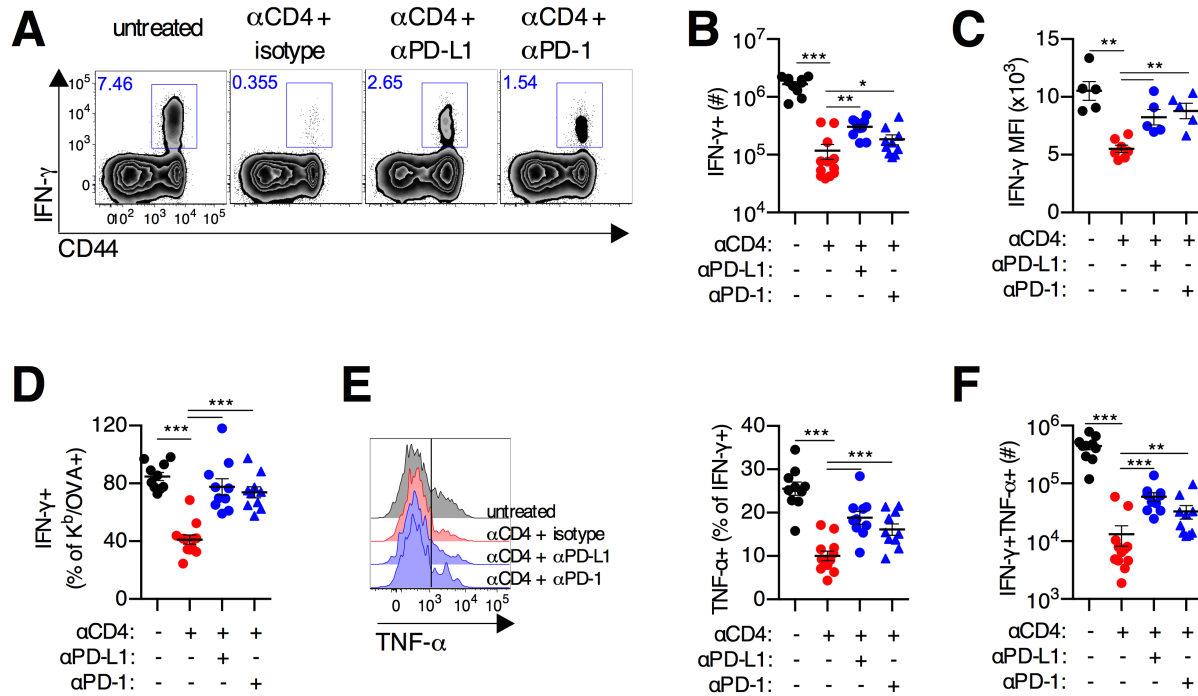


Figure 3.S5. Anti-PD-1 antibody treatment partially rescues the functionality of CD8⁺ T cells primed in the absence of CD4⁺ T cells. C57BL/6 mice were treated with α CD4 antibody or left untreated and immunized intramuscularly with 10^9 vp of Ad5-SIINFEKL-Luc. α CD4 antibody treated mice were treated with either α PD-L1, α PD-1, or isotype control antibody every three days from day 0 to 12. **(A)** Frequency of IFN- γ producing CD8⁺ T cells from the spleen upon *ex vivo* stimulation with OVA₂₅₇₋₂₆₄ peptide. **(B)** Absolute number of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells that produce IFN- γ . **(C)** Per cell expression of IFN- γ as measured by mean fluorescence intensity (MFI). **(D)** Fraction of K^b/OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells capable of producing IFN- γ . **(E)** Frequency of IFN- γ producing CD8⁺ T cells that co-produce TNF- α . **(F)** Absolute number of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells that co-produce IFN- γ and TNF- α . **(A, B, D-F)** Data are pooled from 2 independent experiments (n=5-7 mice/group per experiment). **(C)** Data are representative of two independent experiments (n=5-7 mice/group per experiment). All data are day 14 post-immunization from the spleen. Mean \pm SEM are shown. ***, P<0.001; **, P<0.01; *, P<0.05; Mann-Whitney U test.

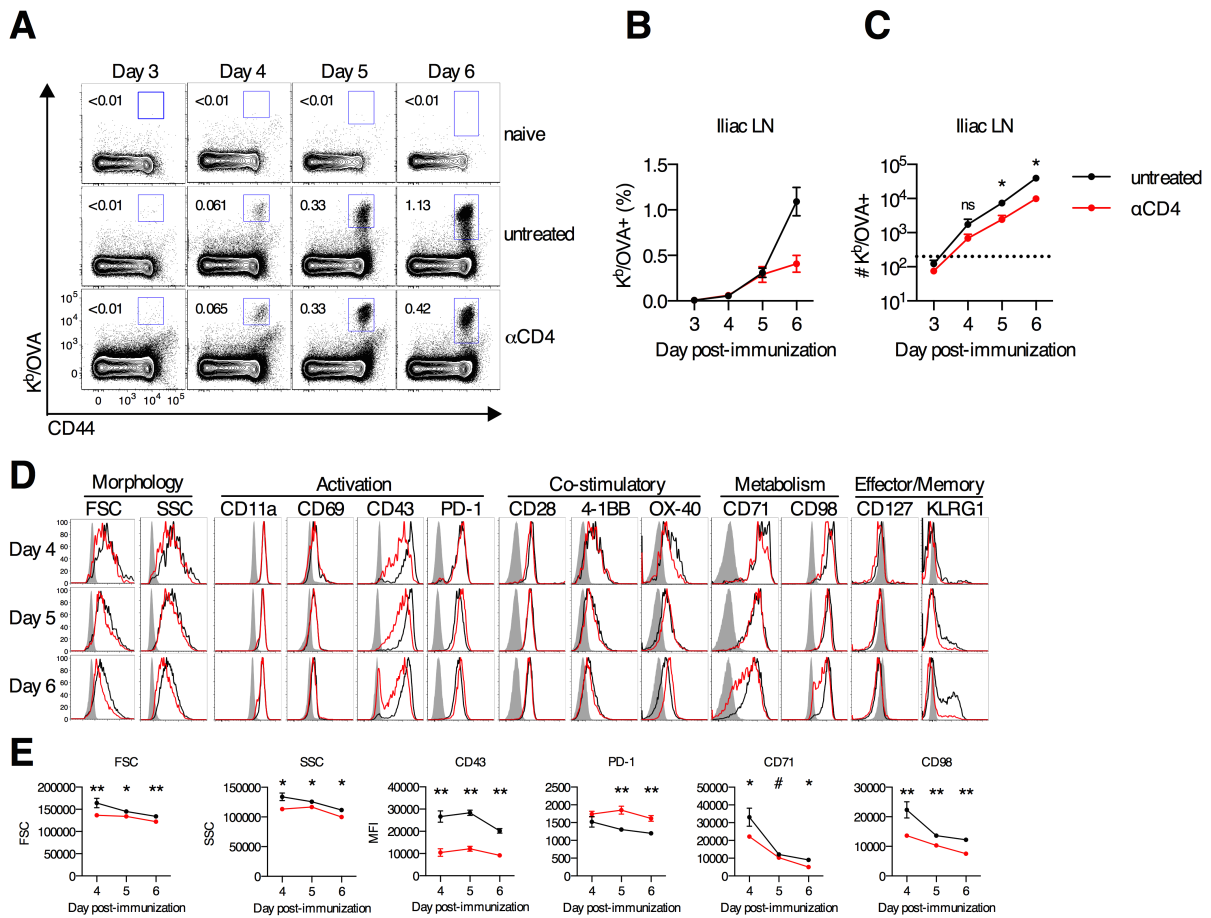


Figure 3.S6. Early phenotype of antigen-specific CD8⁺ T cells primed without CD4⁺ T cell help. C57BL/6 mice were treated with αCD4 antibody or left untreated and immunized intramuscularly with 10⁹ vp of Ad5-SIINFEKL-Luc. **(A)** Representative flow plots of K^b/OVA⁺ CD8⁺ T cells in the iliac LNs. **(B)** Frequency of K^b/OVA⁺ CD8⁺ T cells in the iliac LNs. **(C)** Absolute number of K^b/OVA⁺ CD8⁺ T cells in the iliac LNs. **(D)** Representative flow plots of cellular morphology, activation markers, co-stimulatory receptors, metabolic receptors, and effector/memory markers on K^b/OVA⁺ CD8⁺ T cells in the iliac LNs. **(E)** Group average expression of markers with significant differences from panel D. Data are representative of two independent experiments (n=5 mice/group per experiment). Mean ± SEM are shown. **, P<0.01; *, P<0.05; #, P=0.06; Mann-Whitney U test.